DNA CLONING

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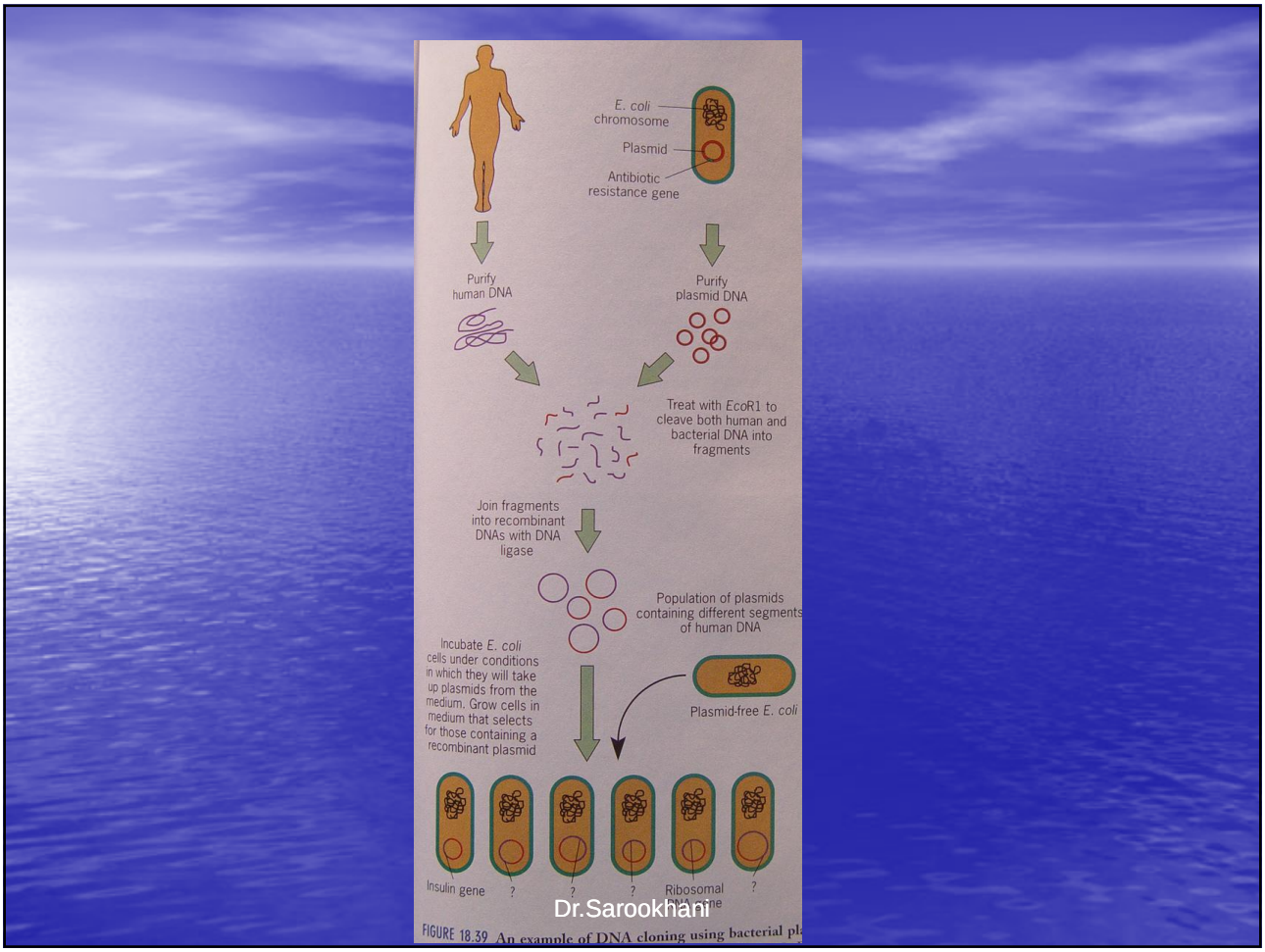
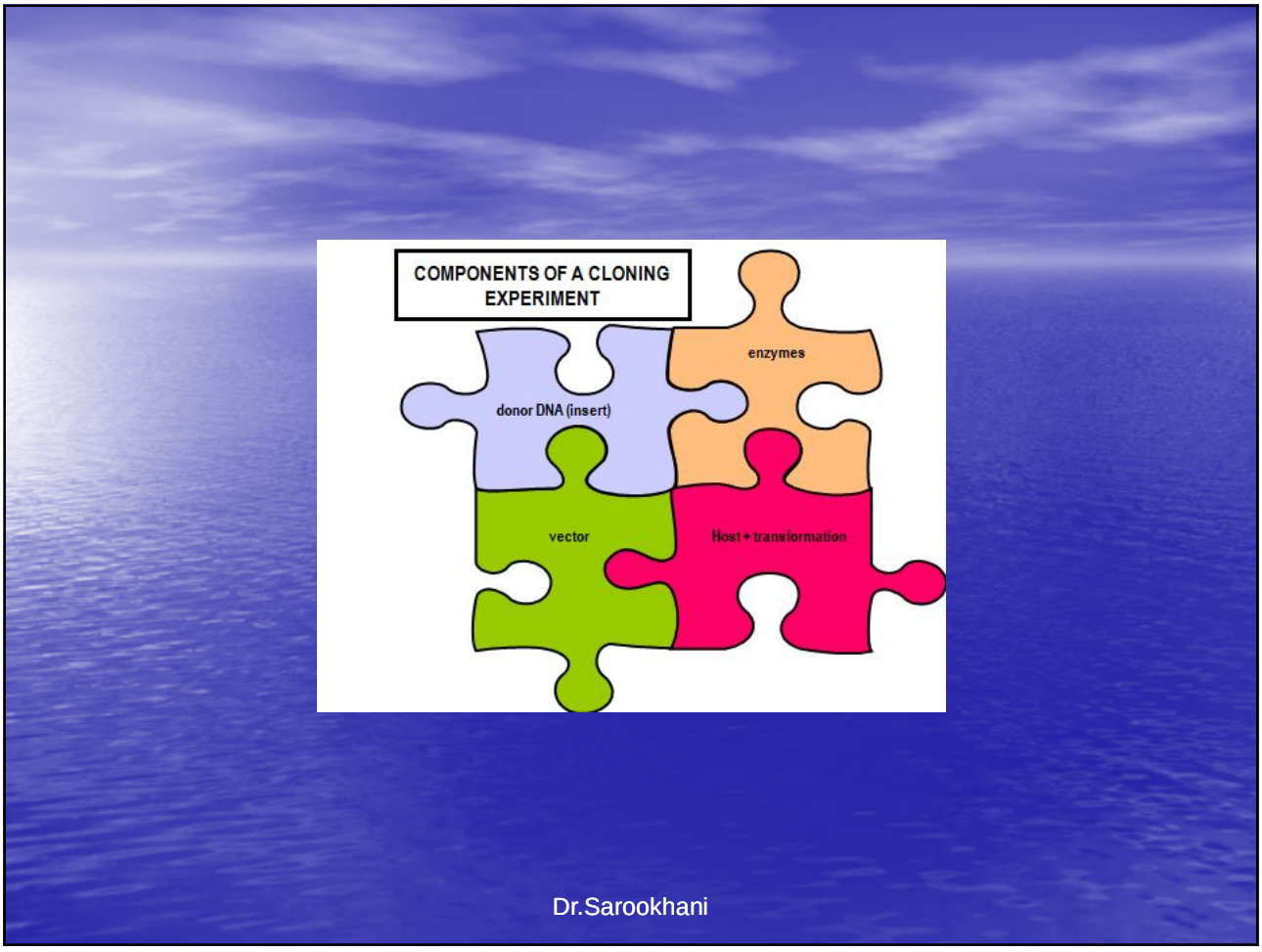


FIGURE 18.39 An example of DNA cloning using bacterial pl


COMPONENTS OF A CLONING EXPERIMENT
enzymes
donor DNA (insert)
vector
Host + transformation
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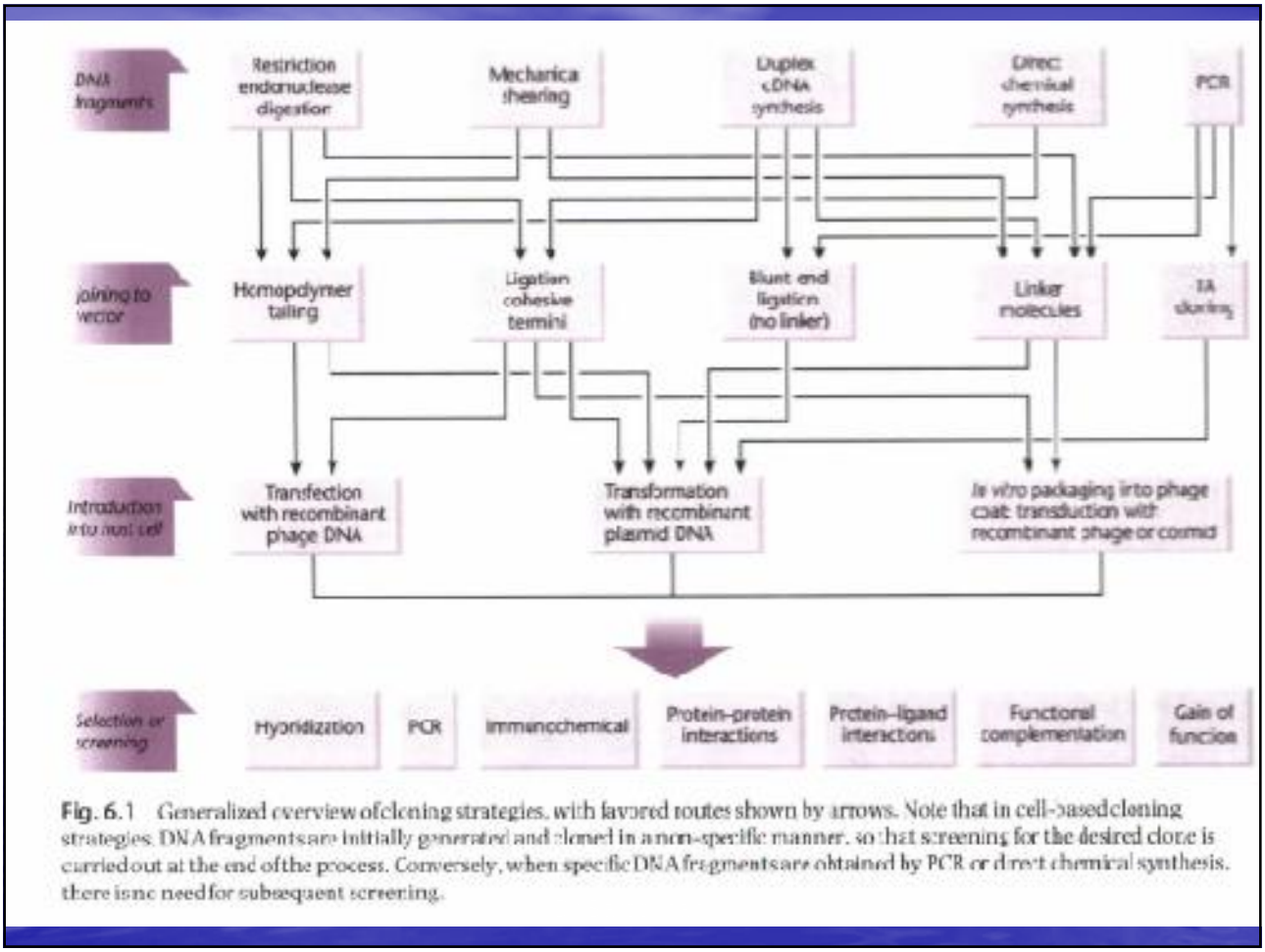

Fig. 6.1 Generalized overview of cloning strategies, with favored routes shown by arrows. Note that in cell-based cloning strategies DNA fragments are initially generated and cloned in a non-specific manner, so that screening for the desired clone is carried out at the end of the process. Conversely, when specific DNA fragments are obtained by PCR or direct chemical synthesis, there is no need for subsequent screening.

WAYS OF GENERATING DNA FRAGMENTS

1. Non-specific generation of truly random fragments (by mechanical shearing or digestion with non-specific nucleases)
2. Through reverse transcription of mRNA into DNA
3. Highly specific amplification of a chosen piece of DNA by PCR
4. The use of synthetic DNA
5. *Restriction endonucleases digestion*

ENZYMES IN CLONING

Cutting and joining DNA

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RESTRICTION ENDONUCLEASES

- Enzymes
- Recognizes a short, symmetrical DNA sequence
- Hydrolyzes/cuts the DNA backbone in each strand
  - Specific site within that sequence
  - Foreign DNA is degraded into short fragments

  - Finger printing

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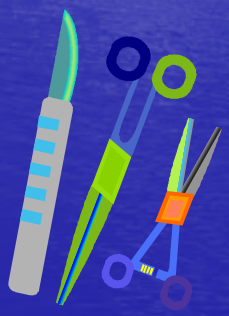
RESTRICTION ENDONUCLEASES

2. Part of the restriction-modification defense mechanism against foreign DNA
3. Basic tools of gene cloning



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RESTRICTION ENDONUCLEASES

3 types

Type I

Type II

Type III

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TYPE II REs

- commonly used in cloning
- recognize and cut within (or immediately adjacent to) specific target sequences
  - generate specific fragments
- a small number
  - cut the DNA at a defined distance (usually only a few bases) away from the recognition site
  - limited applications
- requirement: Mg^{2+}

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CHARACTERIZATION AND IDENTIFICATION

1. The name of the organism from which they are obtained
2. Write in *italics*
   - The first letter of the genus
   - The first two letters of the species name
3. A suffix indicating the specific enzyme from that species

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CHARACTERIZATION AND IDENTIFICATION

- Example:
  - *Pst*I from *Providencia stuartii*
  - *Hae*I, *Hae*II and *Hae*III, three different enzymes, with different specificities from *Haemophilus aegyptius*

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THE PRODUCT OF REs DIGESTION

1. Products with protruding ends known as cohesive or ‘sticky’ ends
   - Fragments with unpaired single-stranded sequences either at the 5’ or 3’ ends

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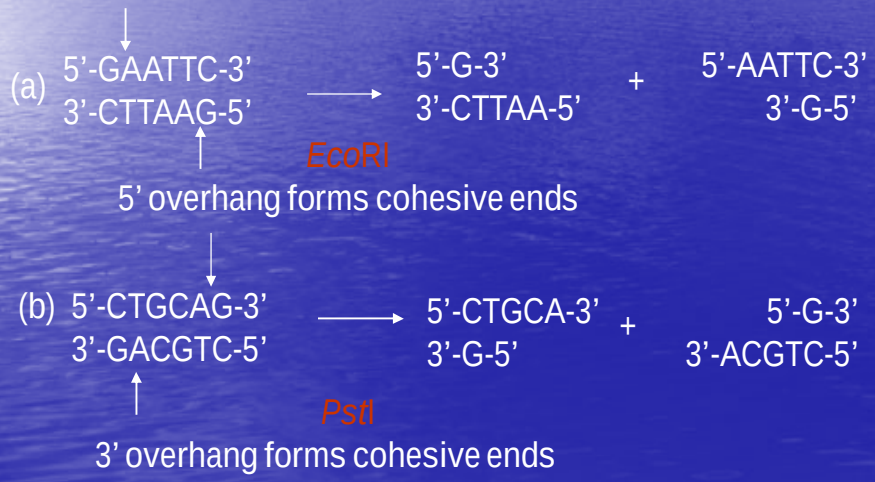
THE PRODUCT OF REs DIGESTION



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THE PRODUCT OF REs DIGESTION

2. Products with blunt ends

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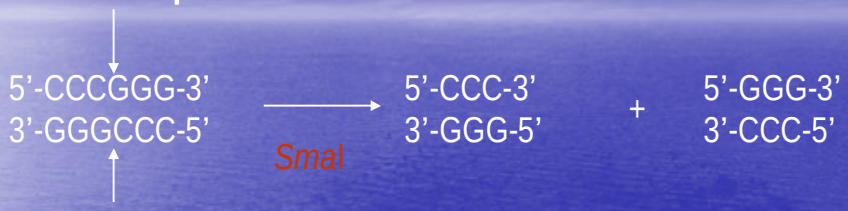
- Example



- Advantage: they can be joined to any other blunt-ended fragment
- Disadvantage: less efficiently ligated

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Enzymes	Recognition site	Number of bases	Ends generated	Source
*Eco*RI	G/AATTC	6	5' sticky	*Escherichia coli* RY13
*Bam*HI	G/GATCC	6	5' sticky	*Bacillus amyloliquefaciens* H
*Bgl*II	A/GATCT	6	5' sticky	*Bacillus globigii*
*Pst*I	CTGCA/G	6	3' sticky	*Providencia stuartii*
*Xma*I	C/CCGGG	6	5' sticky	*Xanthomonas malvacearum*
*Sma*I	CCC/GGG	6	blunt	*Serratia marcescens*
Sau3A	/GATC	4	5' sticky	*Staphylococcus aureus* 3A
*Alu*I	AG/CT	4	blunt	*Arthrobacter luteus*
*Not*I	GC/GGCCGC	8	5' sticky	*Nocardia otitidis-caviarum*
*Pac*I	TTAAT/TAA	8	3' sticky	*Pseudomonas alcaligenes*

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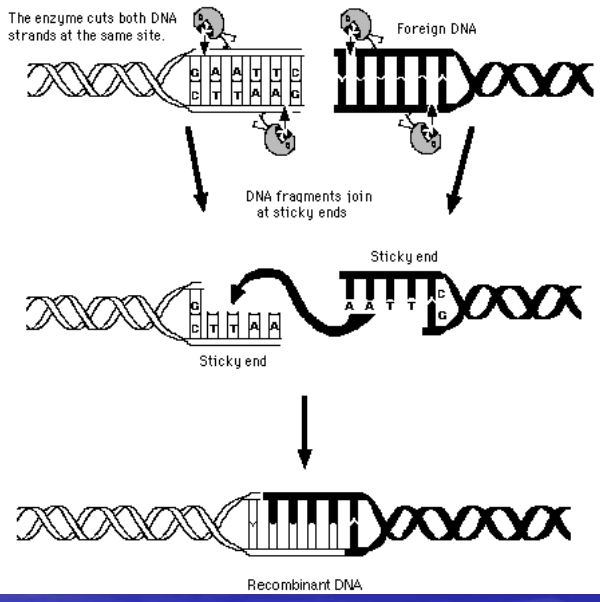
Restriction Enzyme Action of EcoRI



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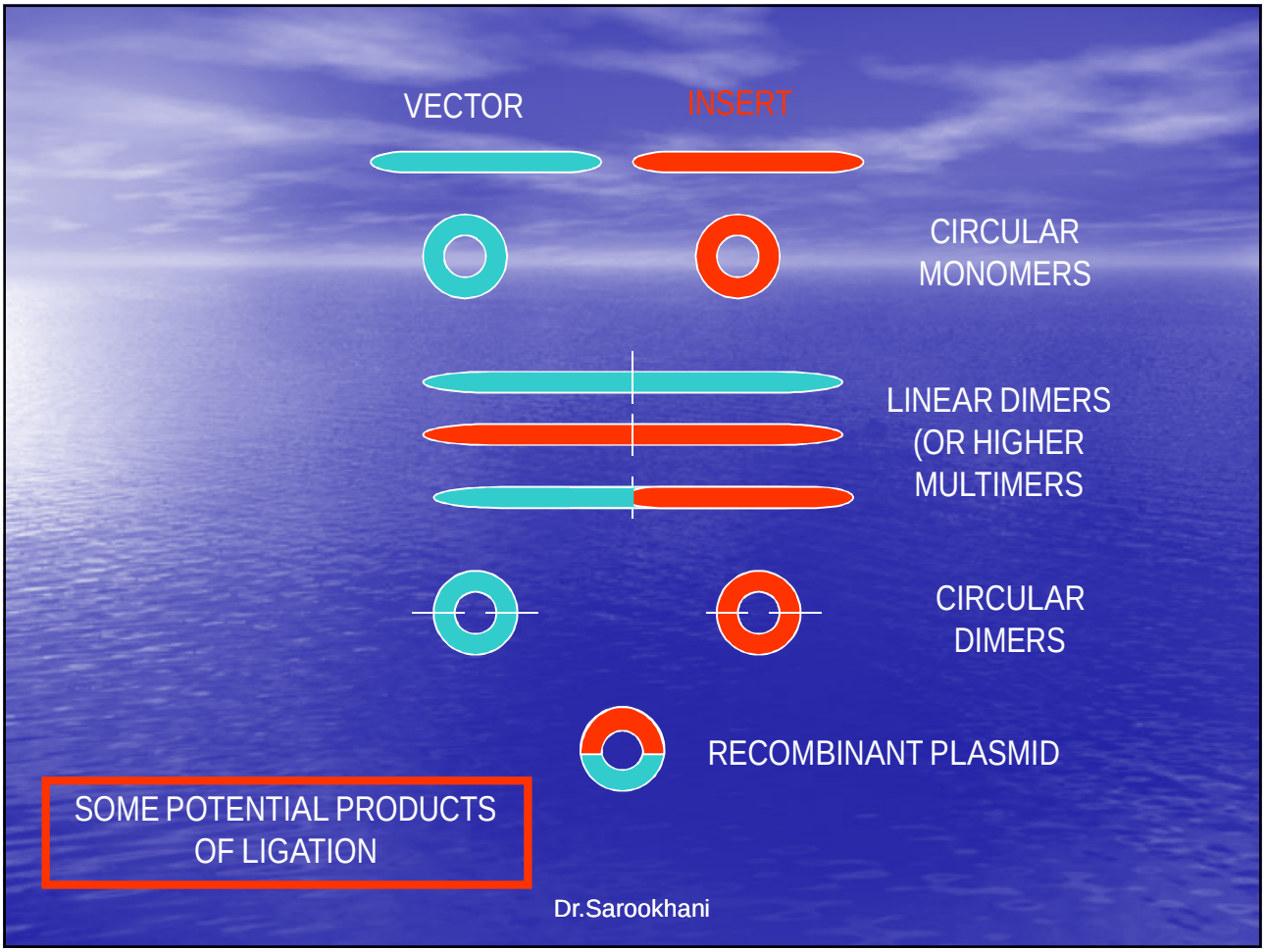
VECTOR
INSERT
CIRCULAR MONOMERS
LINEAR DIMERS (OR HIGHER MULTIMERS
CIRCULAR DIMERS
RECOMBINANT PLASMID
SOME POTENTIAL PRODUCTS OF LIGATION
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OTHER ENZYMES IN CLONING

- Nucleases
- Ligases
- Phosphatase and Kinases
- DNA synthesizing enzymes

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NUCLEASES

Cutting/degrading DNA

ENDONUCLEASES

EXONUCLEASES

MULTIFUNCTIONAL NUCLEASES

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LIGATION

- The next stage in gene cloning
  - joining the DNA fragment to a vector molecule
    - DNA ligase

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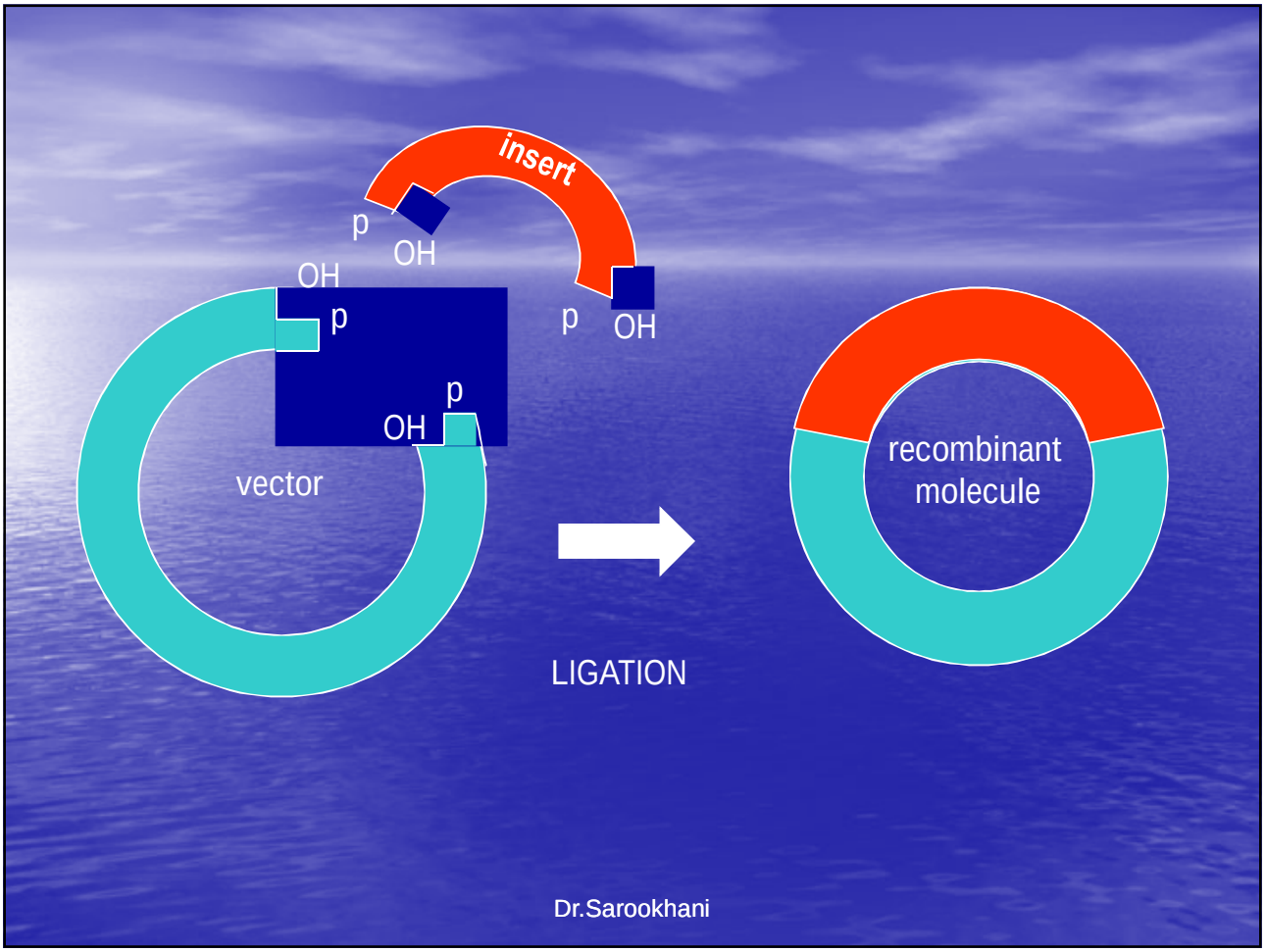
insert
p
OH
OH
p
p
OH
p
OH
vector
LIGATION
recombinant
molecule
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PHOSPHATASES AND KINASES

- Removing or adding respectively phosphate groups
- Examples:
  - alkaline phosphatase: removes the 5' terminal phosphate from a DNA molecule leaving an OH group
  - polynucleotide kinase: adds a phosphate group to a free 5'-terminus
    - reverses the effect of alkaline phosphatase

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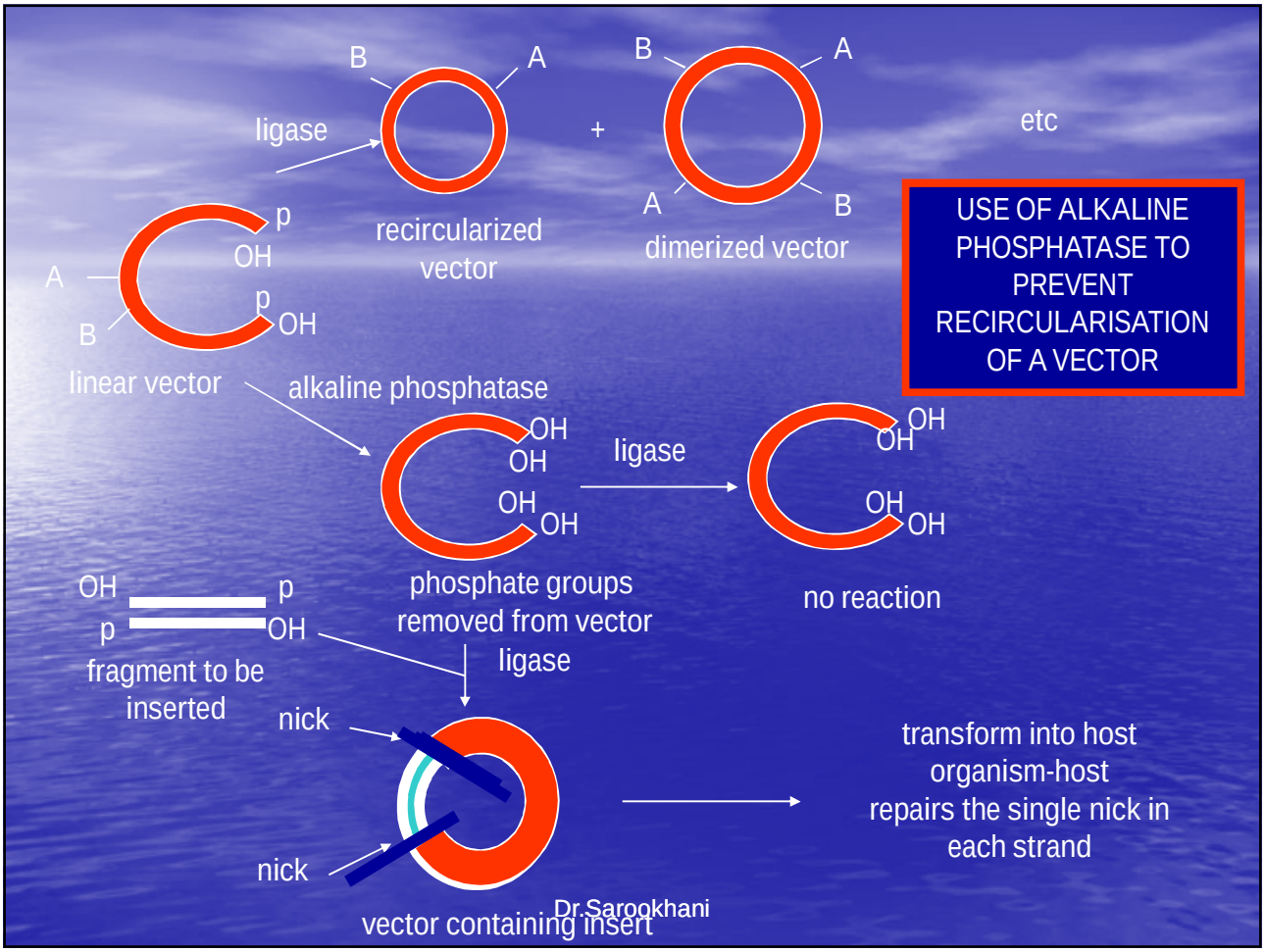
B
A
ligase
recircularized vector
+
B
A
A
B
dimerized vector
etc
USE OF ALKALINE PHOSPHATASE TO PREVENT RECIRCULARISATION OF A VECTOR
p
OH
A
p
B
OH
linear vector
alkaline phosphatase
OH
OH
OH
OH
phosphate groups removed from vector
ligase
OH
OH
OH
OH
no reaction
OH
p
p
OH
fragment to be inserted
ligase
nick
nick
vector containing insert
transform into host organism-host repairs the single nick in each strand
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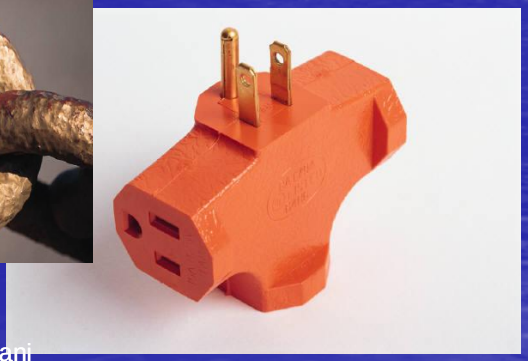
LINKERS AND ADAPTORS

- short synthetic DNA fragments that add new restriction sites to the end of a fragment



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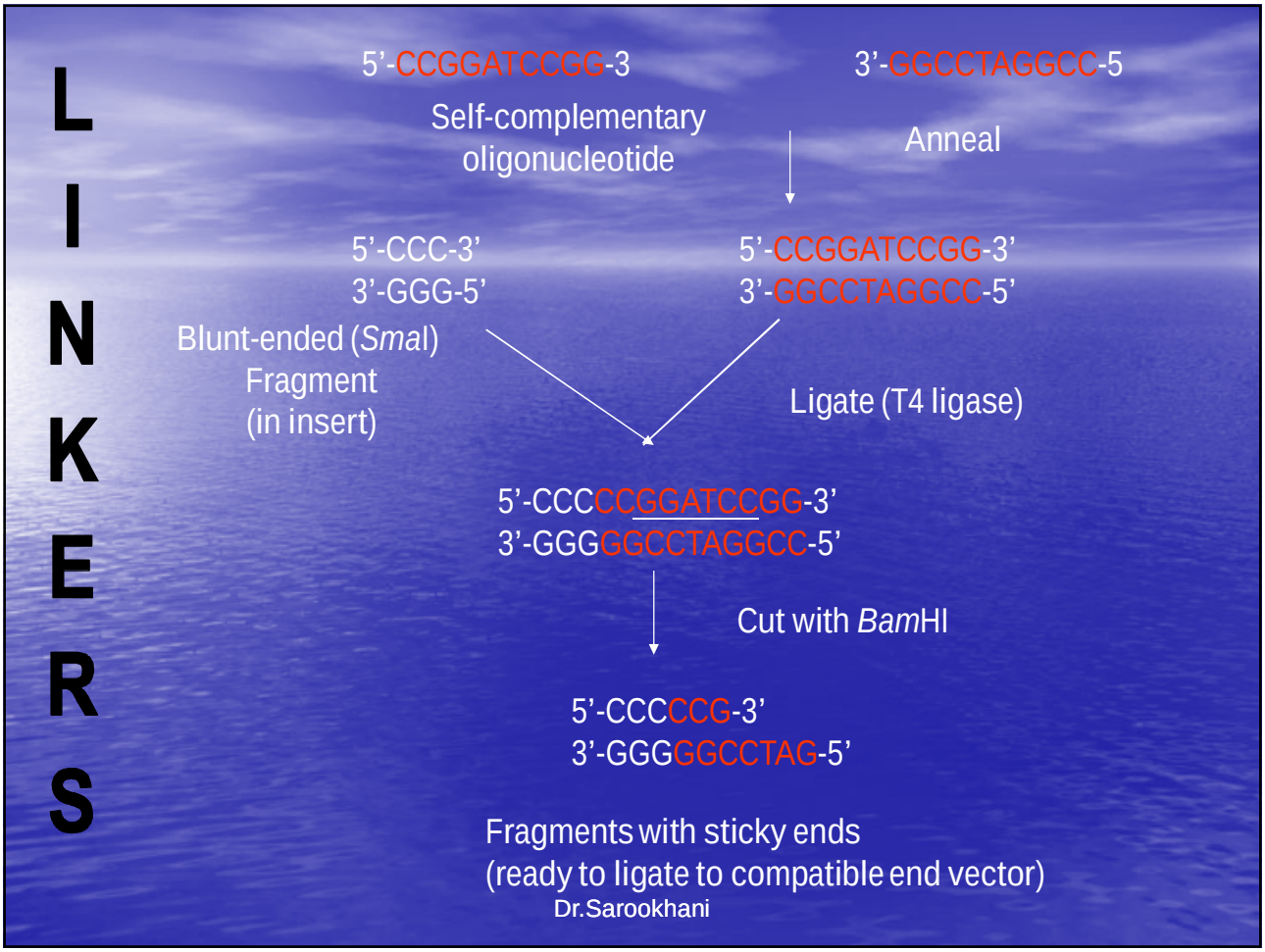
LINKERS
5'-CCGGATCCGG-3
3'-GGCCTAGGCC-5
Self-complementary oligonucleotide
Anneal
5'-CCC-3'
3'-GGG-5'
Blunt-ended (SmaI) Fragment (in insert)
5'-CCGGATCCGG-3'
3'-GGCCTAGGCC-5'
Ligate (T4 ligase)
5'-CCCCCGGATCCGG-3'
3'-GGGGGCCTAGGCC-5'
Cut with BamHI
5'-CCCCCG-3'
3'-GGGGGCCTAG-5'
Fragments with sticky ends
(ready to ligate to compatible end vector)
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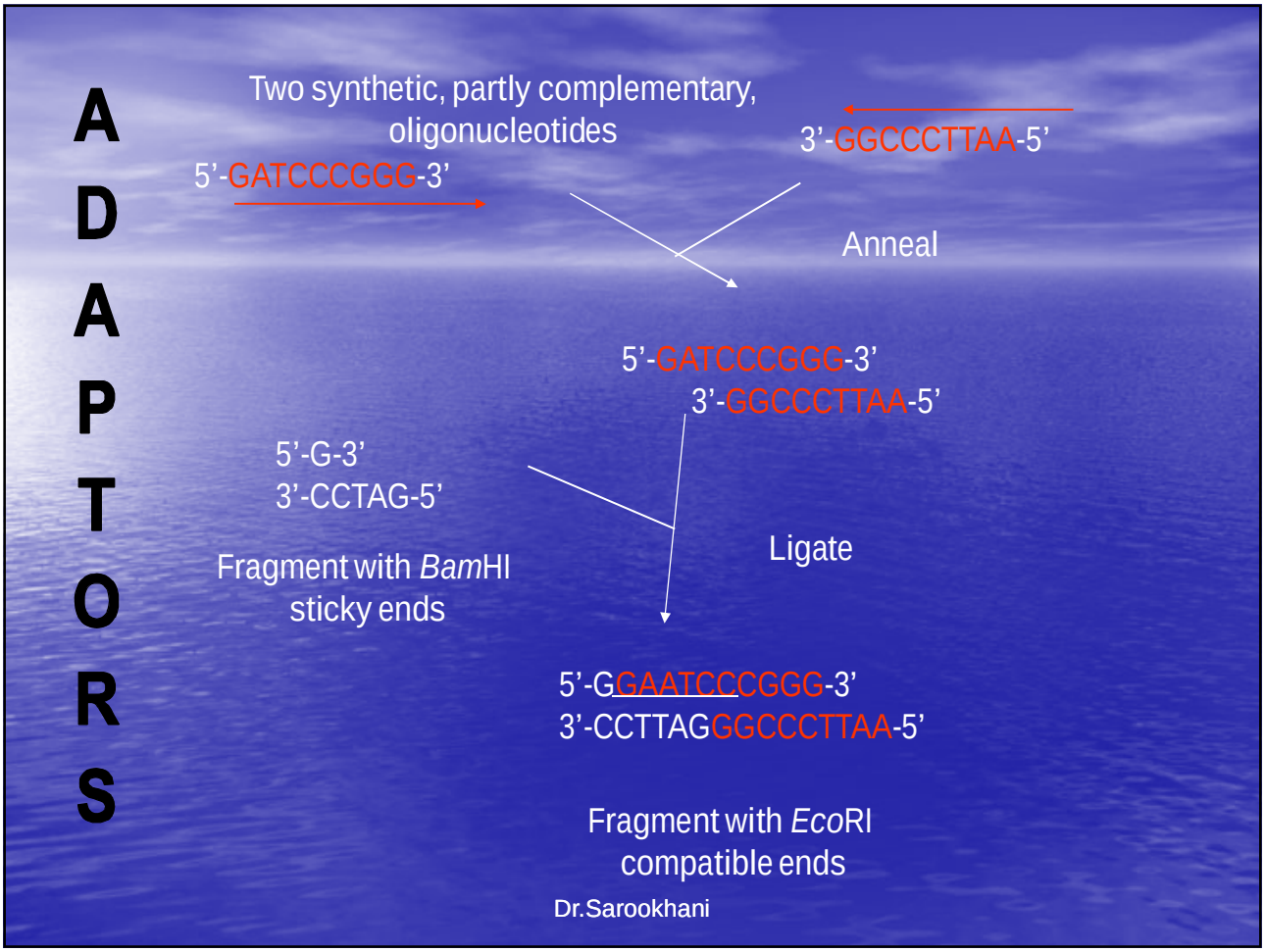
ADAPTORS
Two synthetic, partly complementary, oligonucleotides
5'-GATCCCGGG-3'
3'-GGCCCTTAA-5'
Anneal
5'-GATCCCGGG-3'
3'-GGCCCTTAA-5'
5'-G-3'
3'-CCTAG-5'
Fragment with BamHI sticky ends
Ligate
5'-GGAATCCCGGG-3'
3'-CCTTAGGGCCCTTAA-5'
Fragment with EcoRI compatible ends
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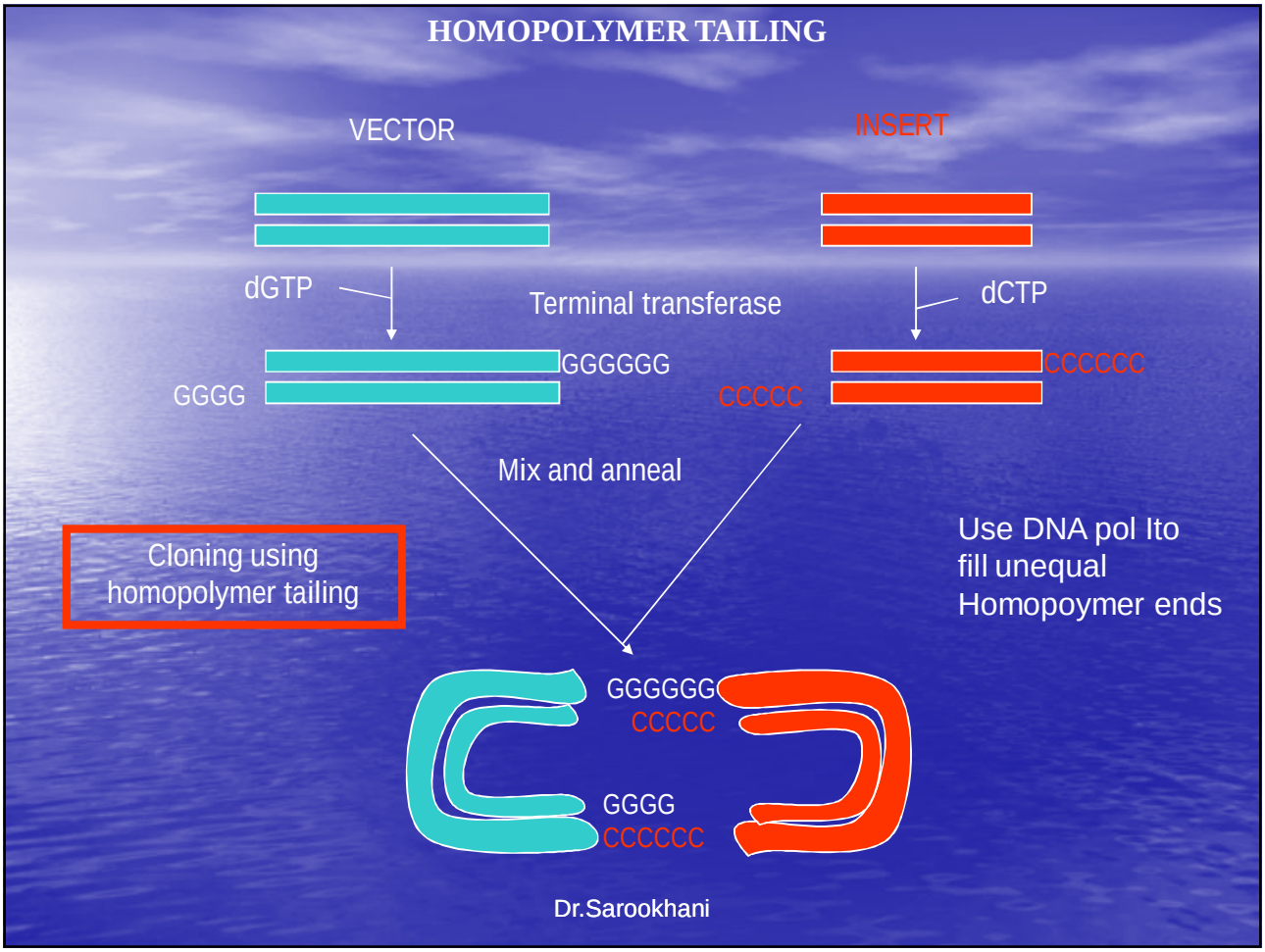
HOMOPOLYMER TAILING
VECTOR
INSERT
dGTP
Terminal transferase
dCTP
GGGGGG
GGGG
CCCCC
CCCCCC
Mix and anneal
Cloning using homopolymer tailing
Use DNA pol Ito fill unequal Homopoymer ends
GGGGGG
CCCCC
GGGG
CCCCCC
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TYPES OF CLONING VECTOR

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TYPES OF CLONING VECTOR (CLONING LIMIT)

Plasmid : 3-5 Kb

Phage : 8-23 Kb

Cosmids : up to 40 Kb

Bacterial Artificial Chromosomes :Up to 1 Mb

Yeast Artificial Chromosomes : Up to 1 Mb(eukaryot)

Retroviral : (for animal cells)

Phasmids (combination of PBR322 & Cosmid of lambda phage)

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TYPES OF CLONING VECTOR (PURPOSE)

- Cloning vector (حامل های کلون)
  - reproduction of the DNA fragment(for gene study due to stability and easy)
- Expression vector
  - expression of certain gene in the DNA fragme

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CHARACTERISTICS OF A GOOD VECTOR

Stable

Easily detected

Easily isolated

Self-replicating

Multiple cloning sites

Small

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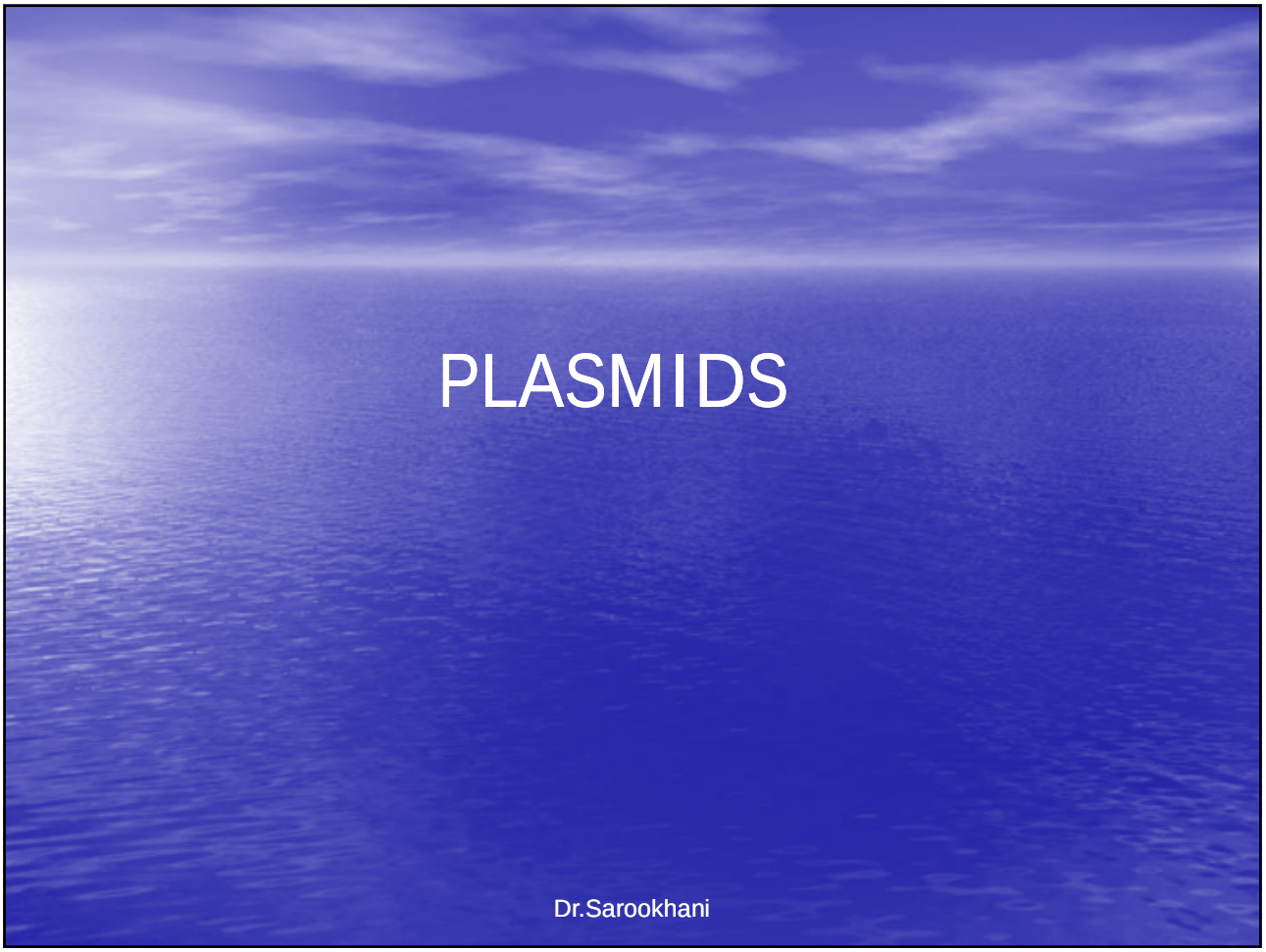
PLASMIDS
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Bacterial cells may contain extra-chromosomal DNA called plasmids.
Plasmids are usually represented by small, circular DNA.

(engineered plasmids can not transfer by itself)

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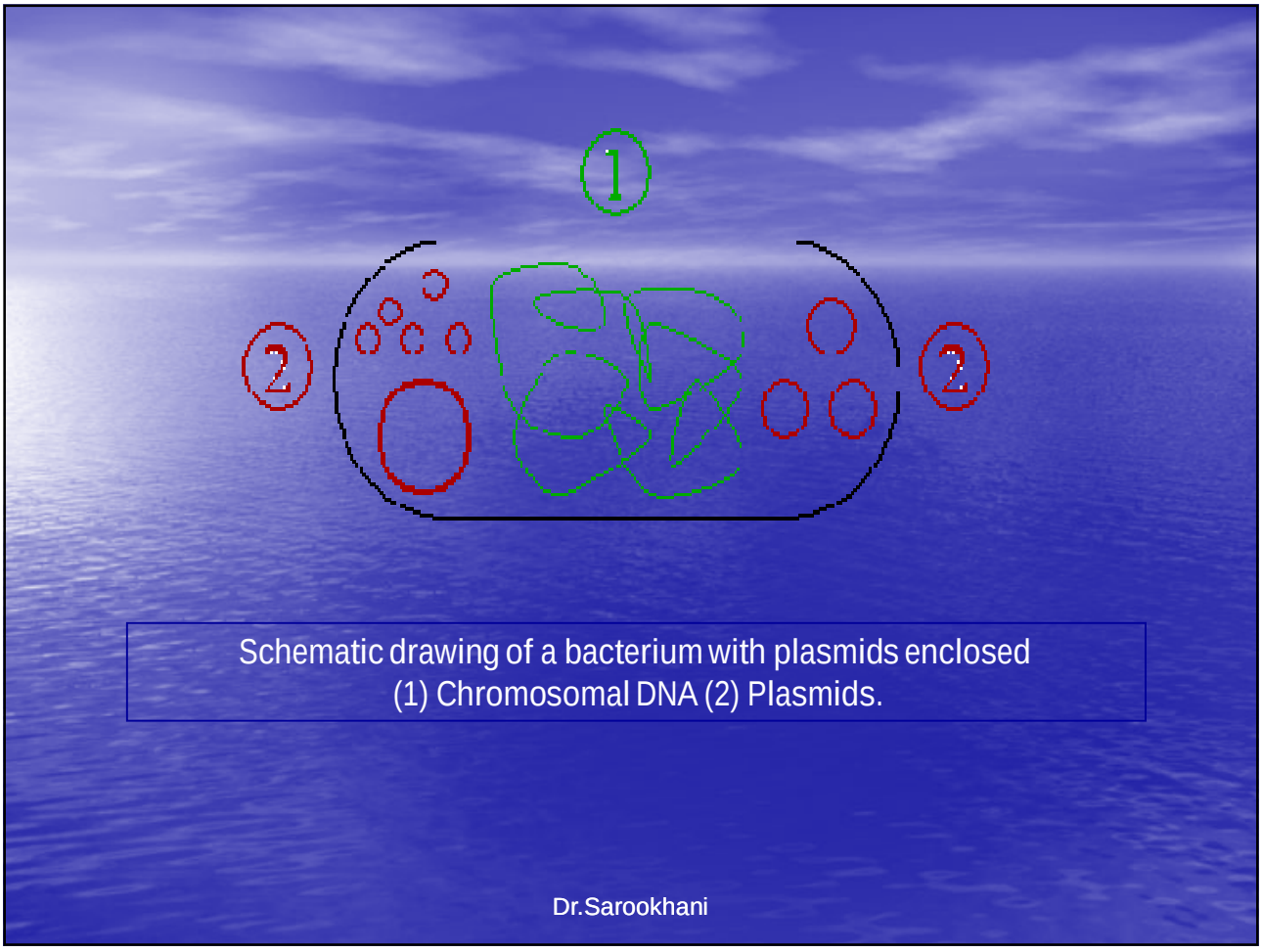
1
2
2
Schematic drawing of a bacterium with plasmids enclosed
(1) Chromosomal DNA (2) Plasmids.
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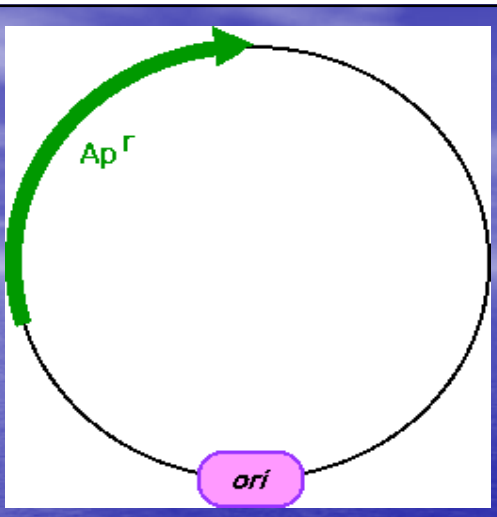

Selective marker is required for maintenance of plasmid in the cell. Because of the presence of the selective marker the plasmid becomes useful for the cell. Under the selective conditions, only cells that contain plasmids with selectable marker can survive. Commonly, genes that confer resistance to various antibiotics are used as selective markers in cloning vectors. For example, genes that render cells resistant to ampicillin, neomycin, or chloramphenicol are among commonly used selective markers.

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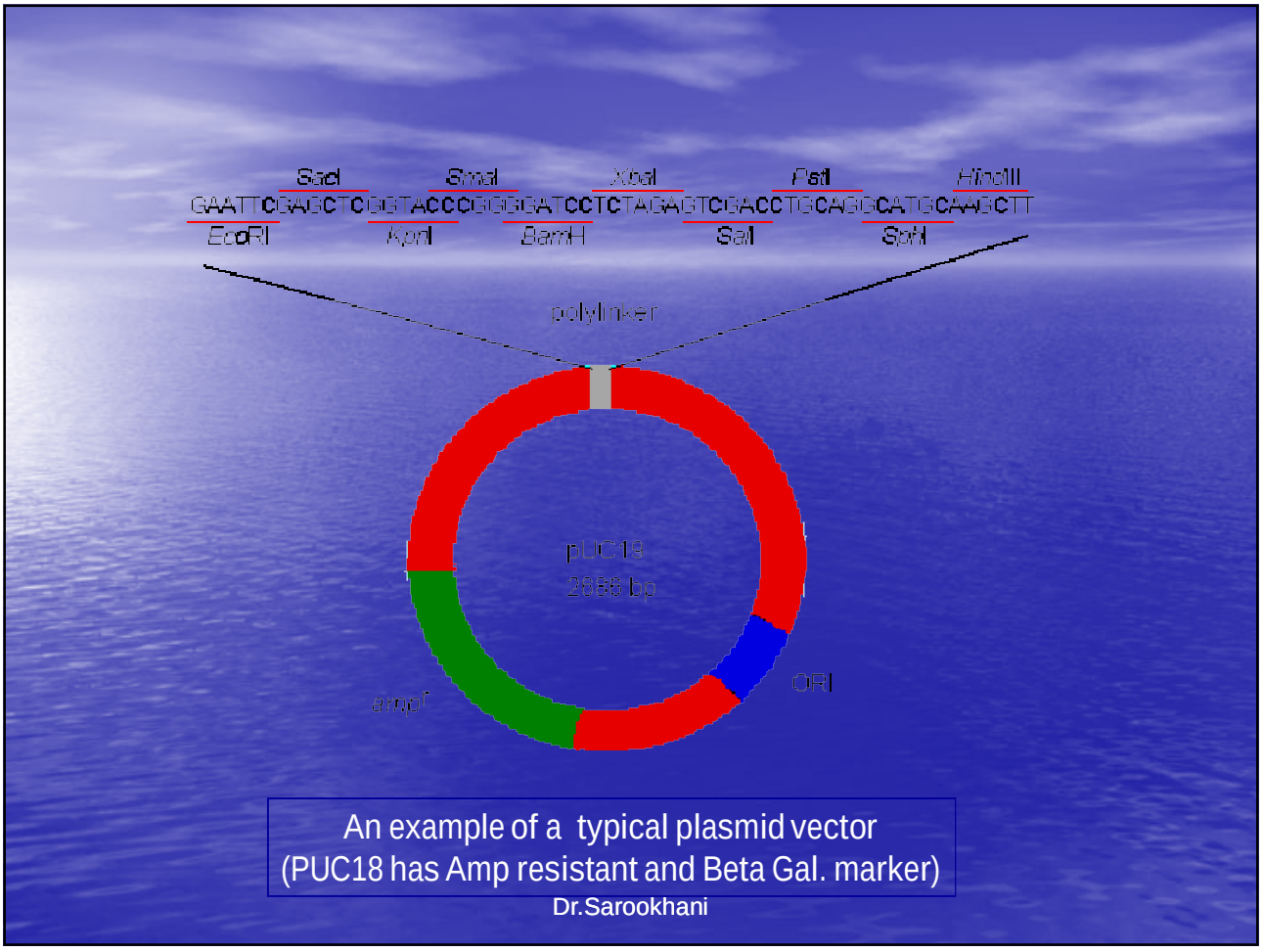

An example of a typical plasmid vector
(PUC18 has Amp resistant and Beta Gal. marker)

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Production of Whole Genomic library by phages or plasmids

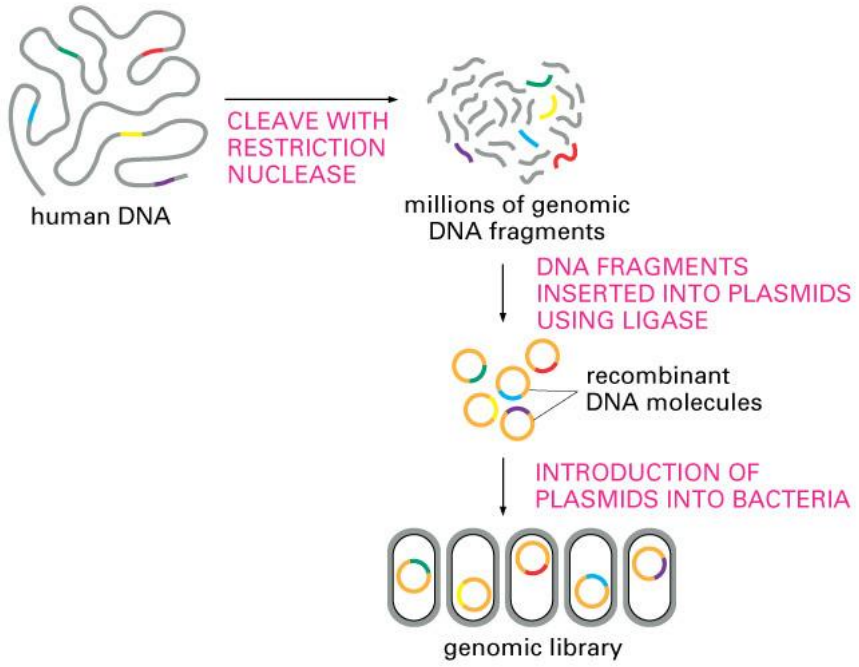
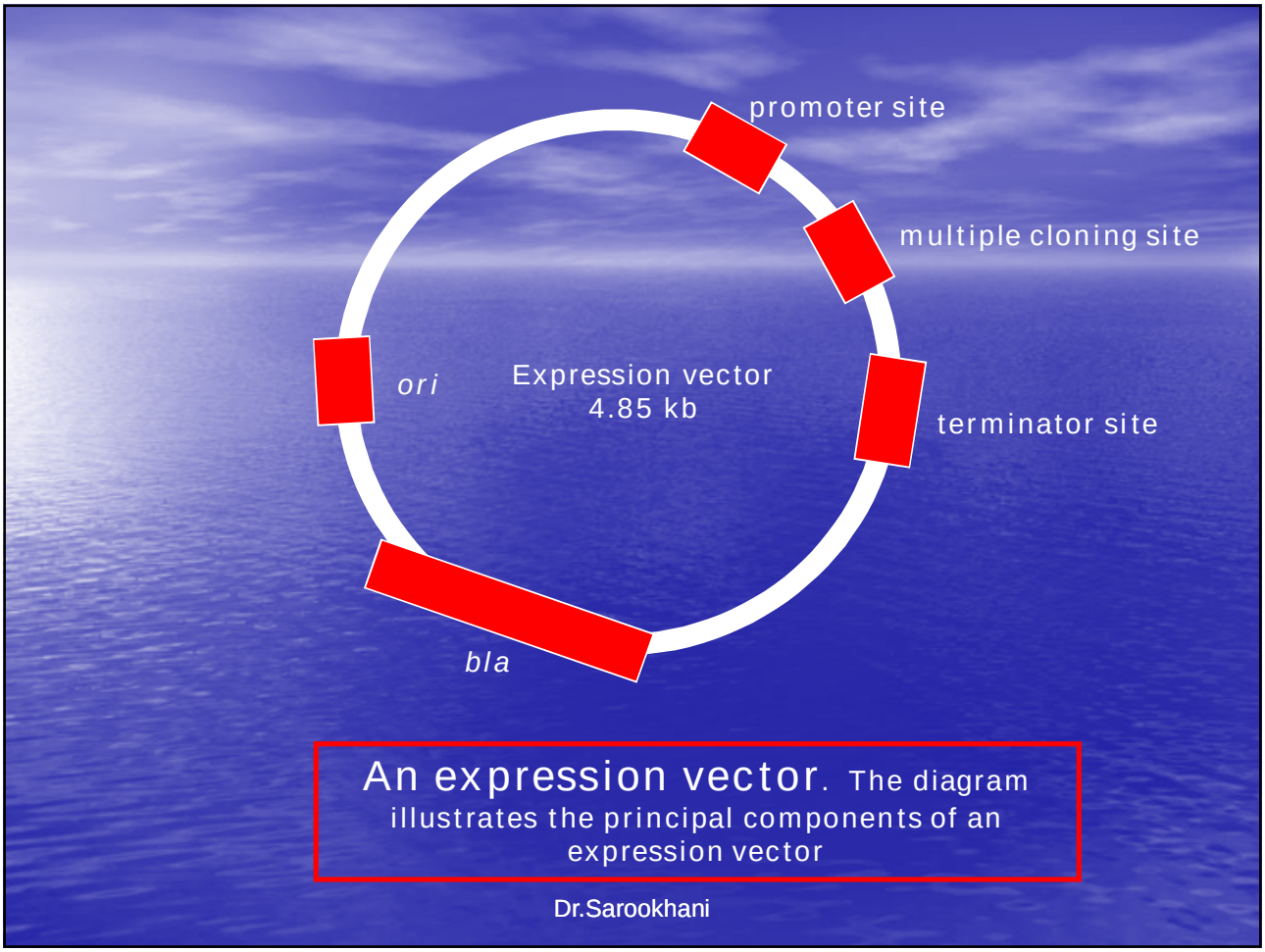


Figure 10-23 Essential Cell Biology, 2/e. (© 2004 Garland Science)



An expression vector. The diagram illustrates the principal components of an expression vector

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Bacteriophage λ

(as a cloning vector)

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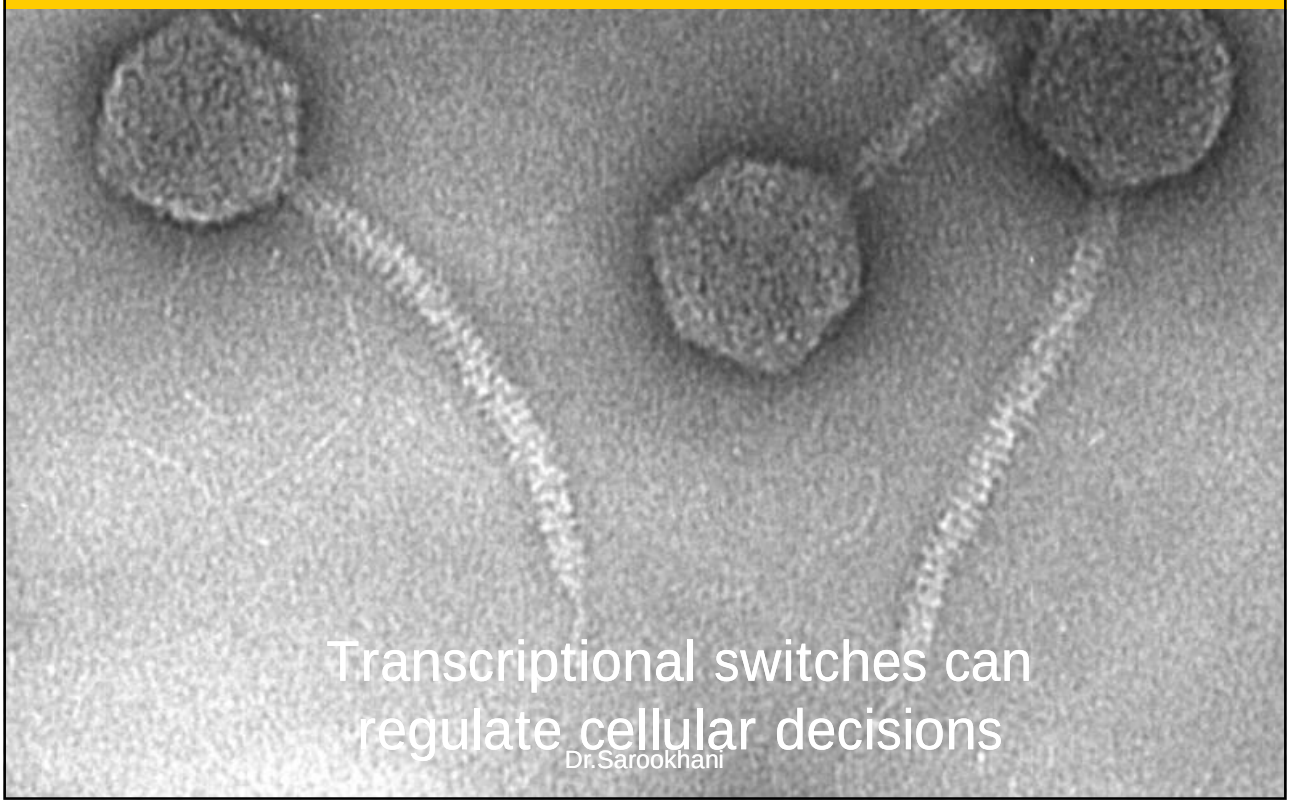
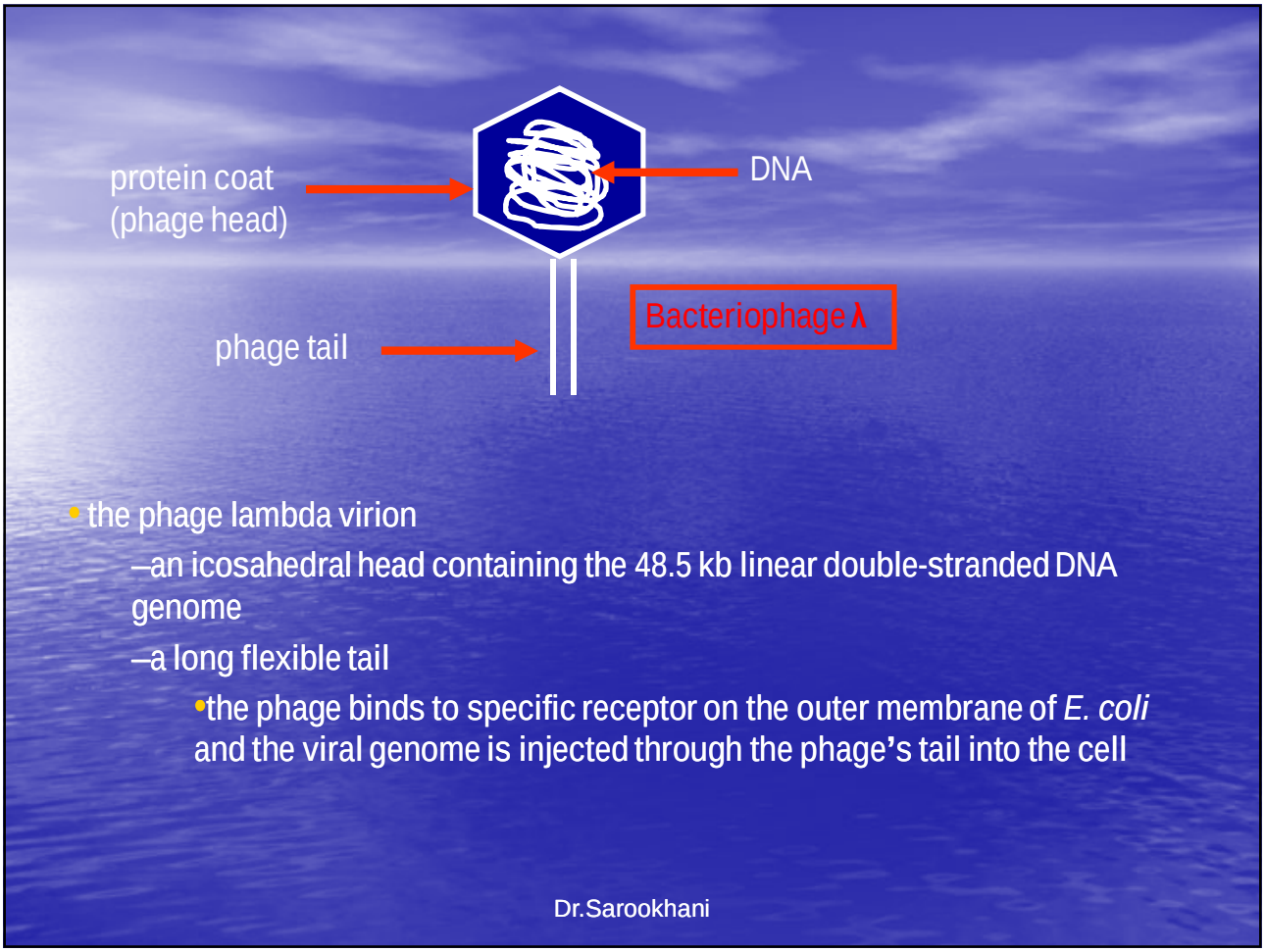
Bacteriophage lambda (I)





- the phage lambda virion
  - –an icosahedral head containing the 48.5 kb linear double-stranded DNA genome
  - –a long flexible tail
    - the phage binds to specific receptor on the outer membrane of *E. coli* and the viral genome is injected through the phage's tail into the cell

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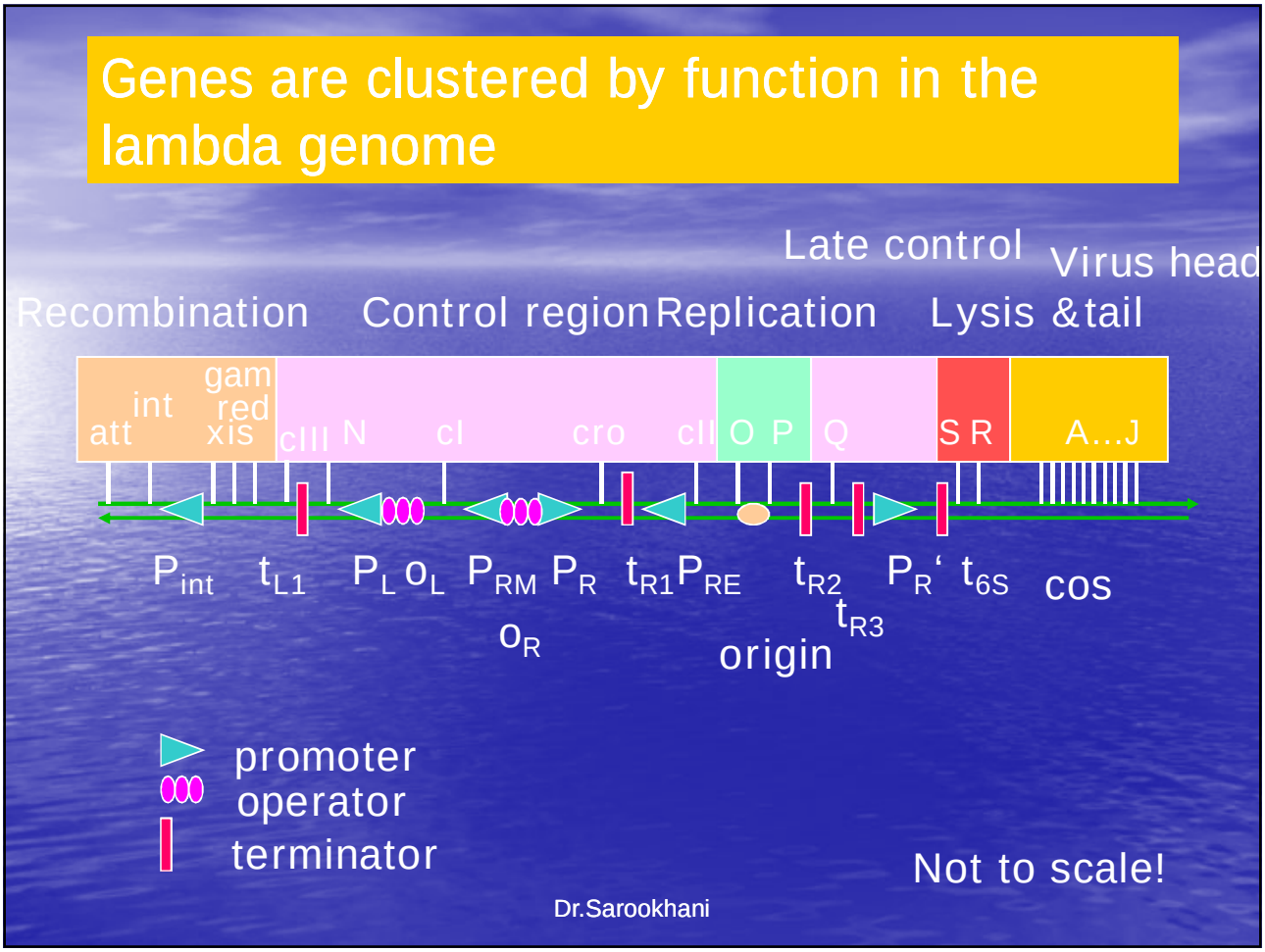
Genes are clustered by function in the lambda genome



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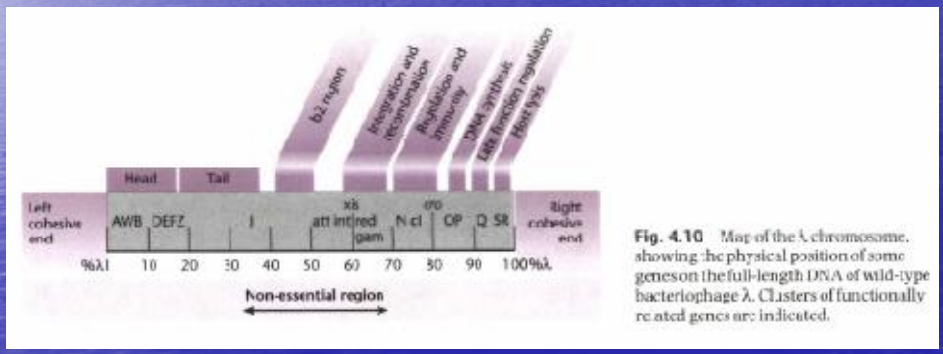

Fig. 4.10 Map of the λ chromosome, showing the physical position of some genes on the full-length DNA of wild-type bacteriophage λ. Clusters of functionally related genes are indicated.

Cos site for circularization of genome whithin the E.coli

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Lysis or Lysogeny

Lysis: Infection by phage produces many progeny and breaks open (lyses) the host bacterium

Lysogeny: After infection, the phage DNA integrates into the host genome and resides there passively

No progeny

No lysis of the host

Can subsequently lyse (lysogeny)

Bacteriophage lambda can do either.

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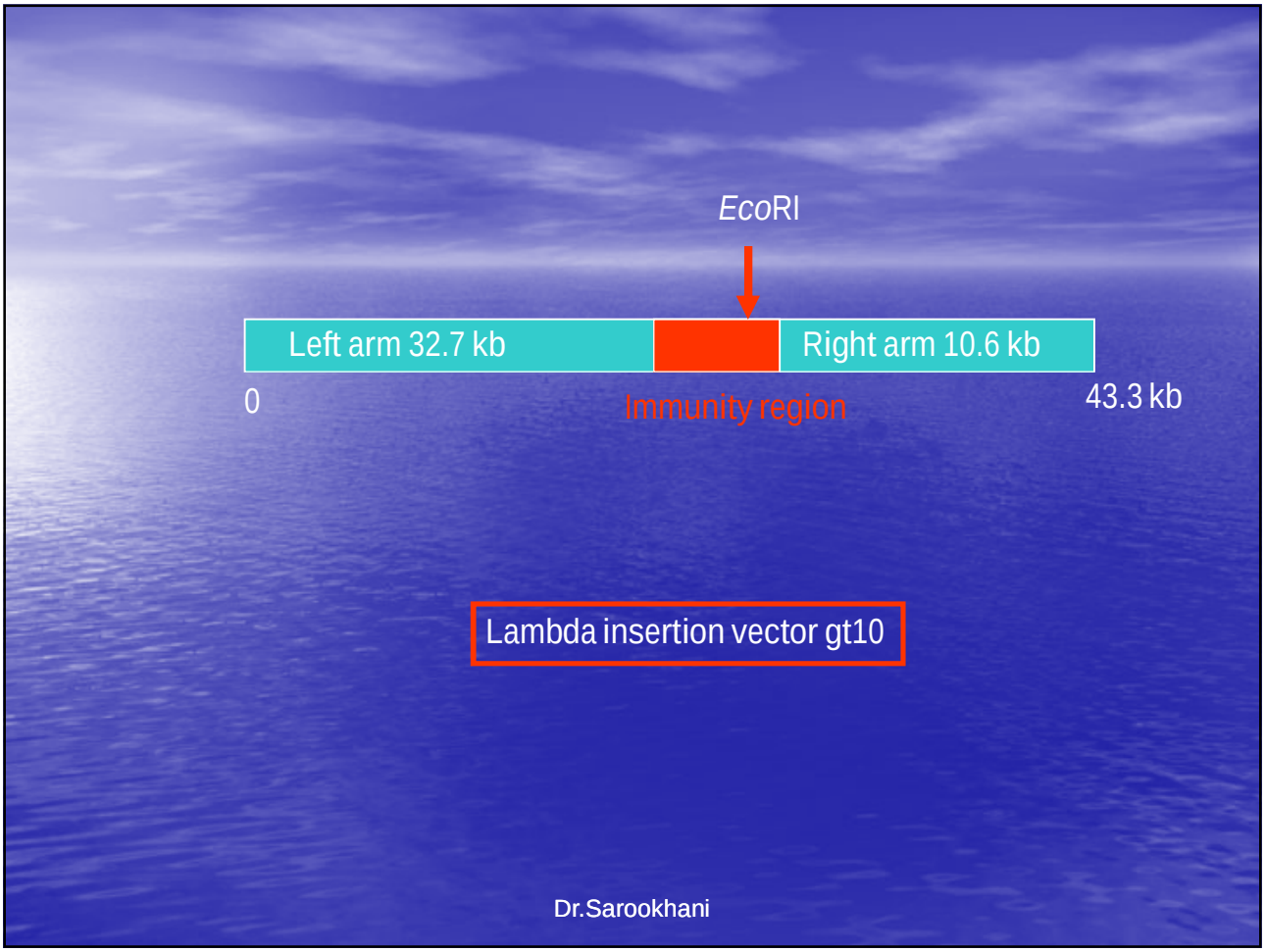
EcoRI
Left arm 32.7 kb
Right arm 10.6 kb
0
Immunity region
43.3 kb
Lambda insertion vector gt10
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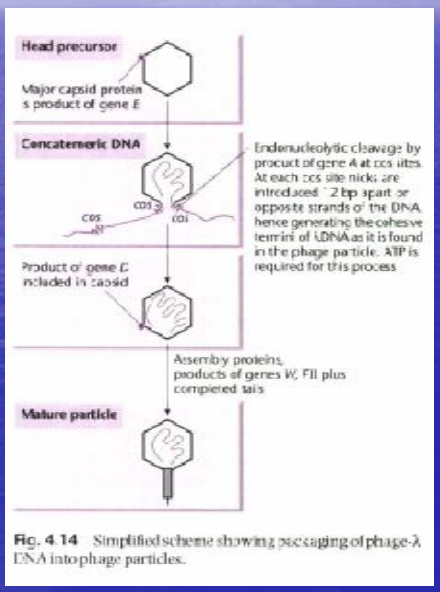

Fig. 4.14 Simplified scheme showing packaging of phage-λ DNA into phage particles.

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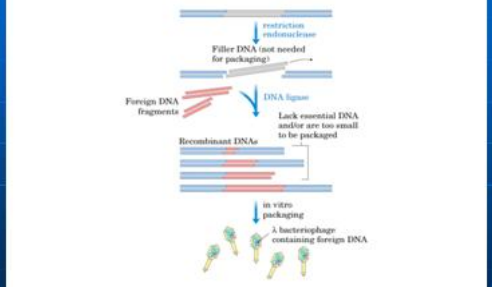
Vector construct



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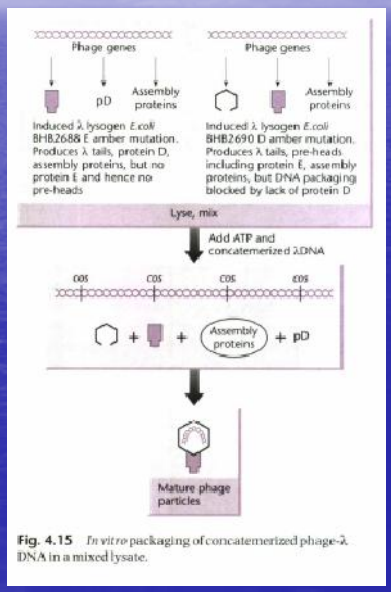

Fig. 4.15 *In vitro* packaging of concatemerized phage-λ DNA in a mixed lysate.

In vitro packaging by cell lysate

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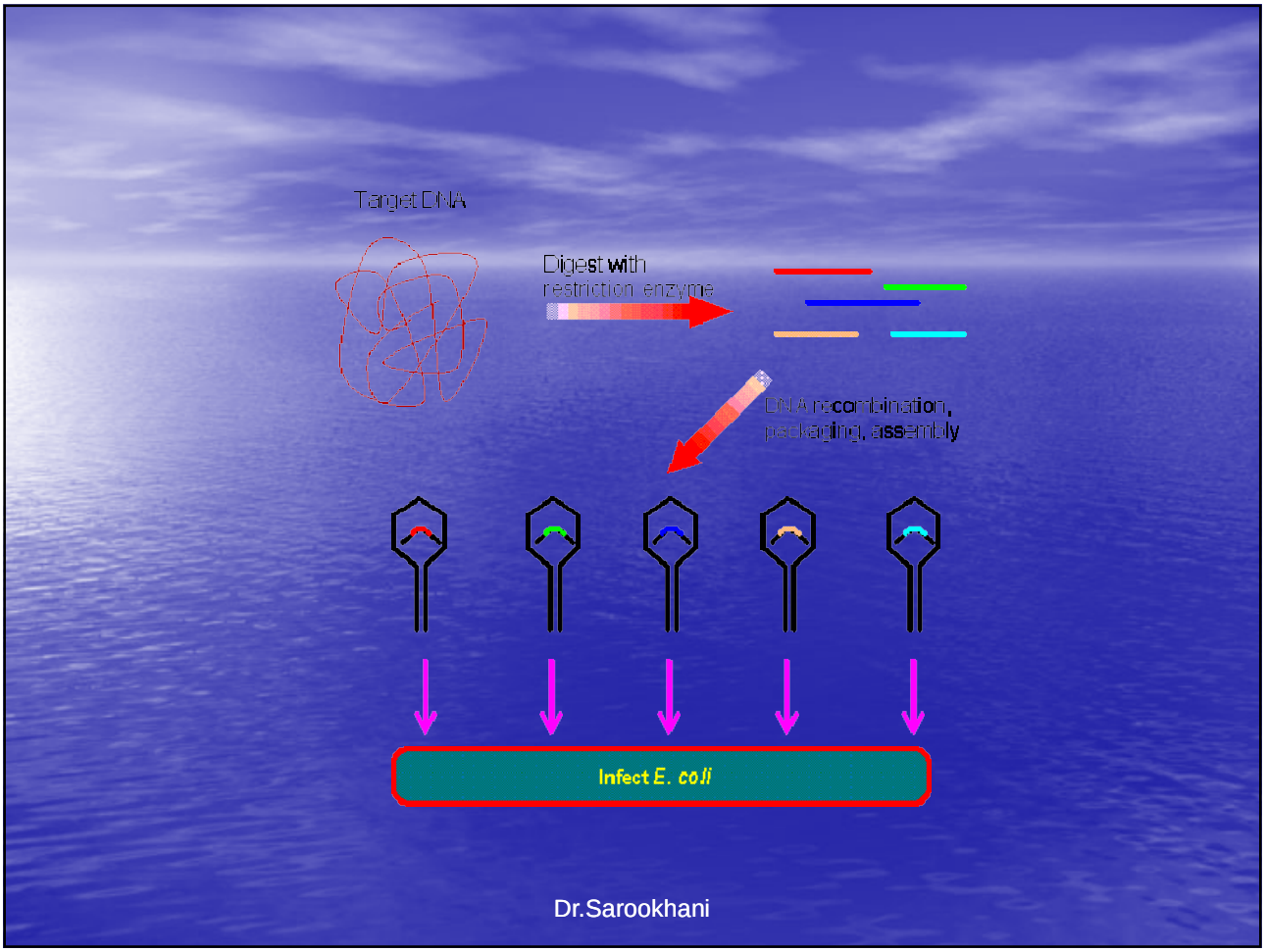
Target DNA
Digest with
restriction enzyme
DNA recombination,
packaging, assembly
Infect E. coli
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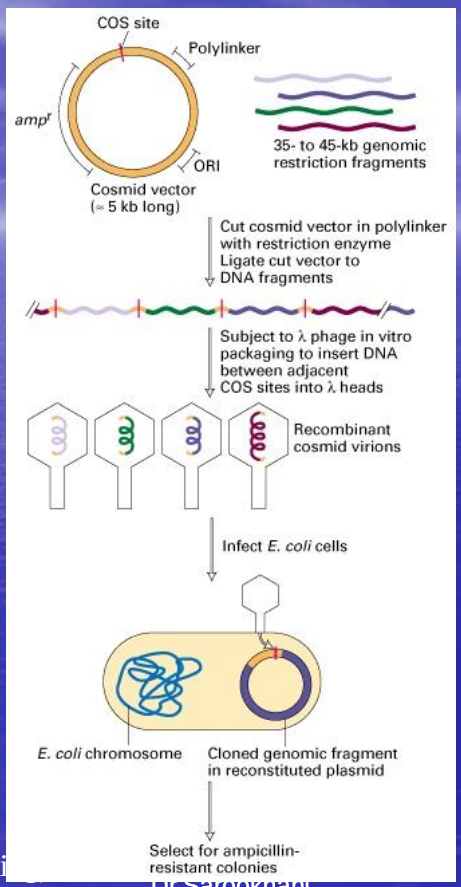

(Used for chromosomal walki

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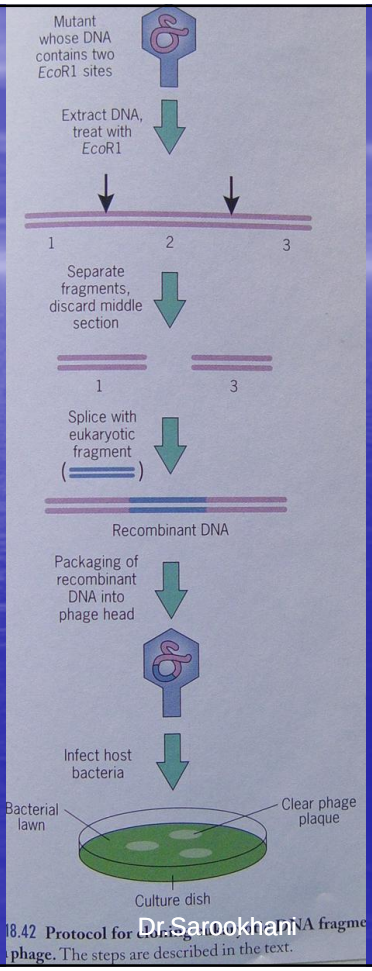

18.42 Protocol for [illegible] **NA fragme**
phage. The steps are described in the text.

COSMIDS

- a plasmid containing a *cos* site
  - *cos* site: the sequence of bases of bacteriophage lambda that is cut asymmetrically during packaging, generating an unpaired sequence of 12 bases at each end of the phage DNA

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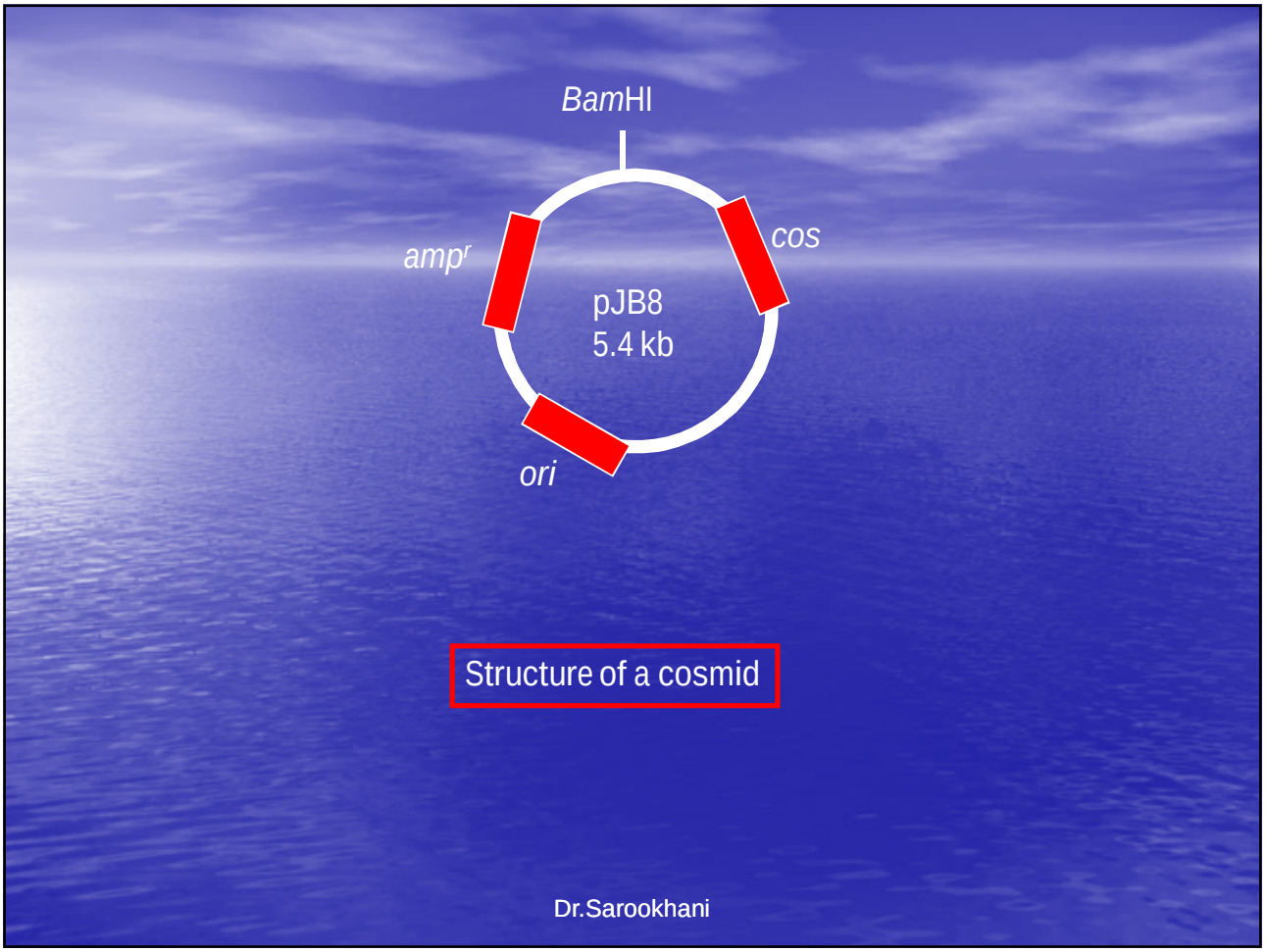
BamHI
cos
ampr
pJB8
5.4 kb
ori
Structure of a cosmid
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YEAST ARTIFICIAL CHROMOSOMES (YACs) AND BACTERIAL ARTIFICIAL CHROMOSOMES (BACs)

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YACs AND BACs

- vectors capable of carrying very large cloned fragments
  - can accommodate genomic DNA fragments of more than 1 Mb (1 Mb=1000kb)
    - cloning the entire human genes
    - example: the cystic fibrosis gene ~ 250 kb
  - mapping the large-scale structure of large genome
    - example: the human genome project

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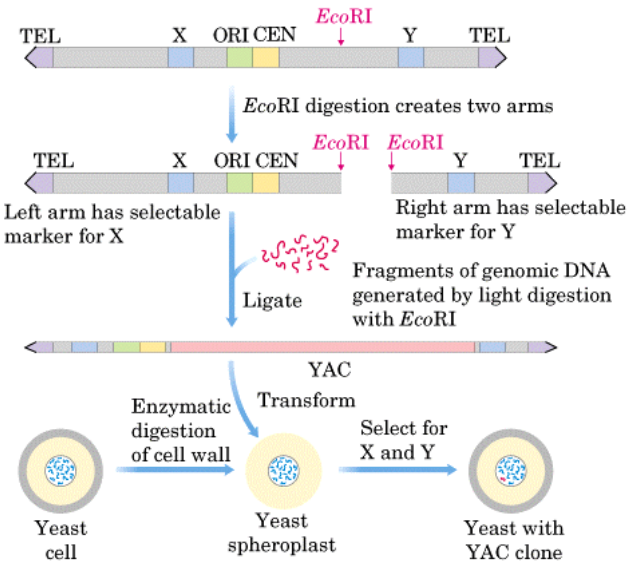
Vector transfer in yeast



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HOST

1. *E. coli*

well understood and conveniently manipulable organism

2. Bacillus subtillis
3. *Saccharomyces cerevisiae*

– frequently used eukaryotic host

– retains many of the convenient features of *E. coli*

4. *Picchia pastoris* (another yeast)
5. Cultured cells of *Spodoptera frugiperda* (an insect)

6- Dorosophila for use with baculoviruses

7- cell lines

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Summary of E.coli Elite host Cells

Product	Genotype	Application
Elite Competent Cells (XL1-Blue)	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac[F'proAB $lacI^qZ\Delta M15$ Tn10 (Tet^r)]	Suitable for blue-white color screening and routine cloning applications.
Elite Competent Cells [BL21(DE3)]	F^- ompT $hsdS_\beta$ ($r_\beta^-m_\beta^-$) dcm galλ(DE3)	Appropriate host for recombinant protein expression using T7-based expression vectors.
Elite Competent Cells (DH5α)	endA1 recA1 relA1 relA1 gyrA96 hsdR17(r_k^-,m_k^+) phoA supE44 thi-1Δ(lacZYA-argF)U169Φ80 Δ(lacZ) $M15F^-$	Suitable for cloning with large plasmid and cDNA library construction, and also allowing blue-white colonies selection.
Elite Competent Cells (JM109)	$e14^-$($McrA^-$)recA1 endA1 gyrA96 thi-1 hsdR17($r_k^-m_k^+$)supE44 relA1 Δ(lac-proAB) [F'traD36 proAB $lacI^qZ\Delta M15$]	Appropriate for blue-white color and robotic screening (fast growing strain, forming visible colonies within 8~10 hours).

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Introducing vectors into host cells

- Transformation
- transfection
- Electroporation
- Nucleic acid gun
- Protoplast fusion
- Micro injection of DNA

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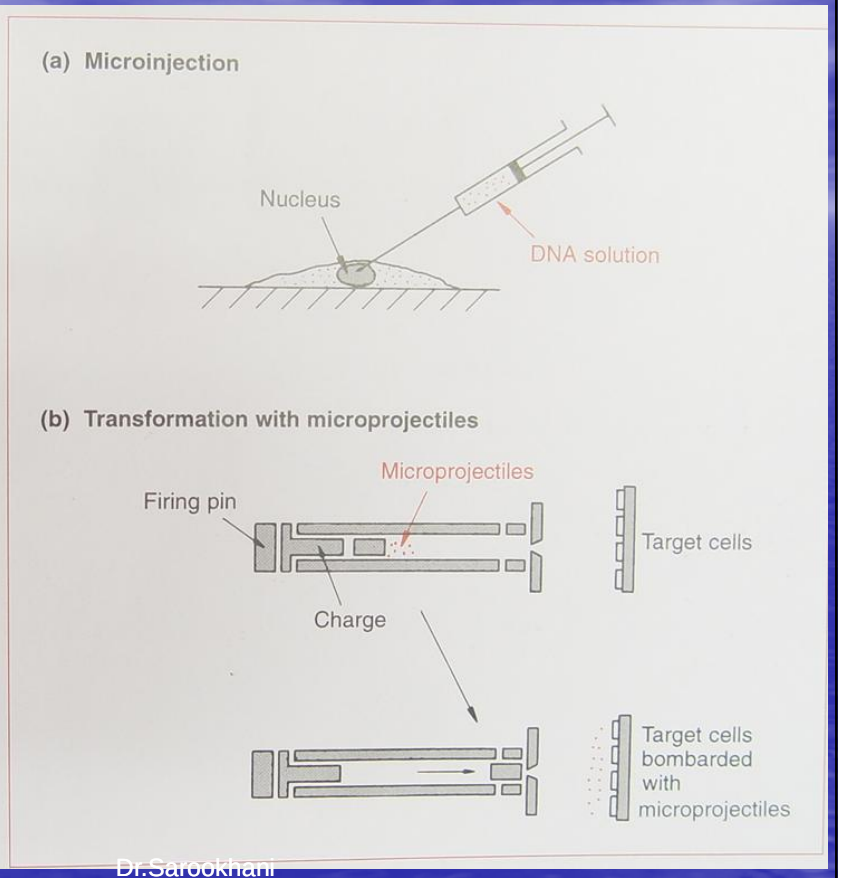
Figure 5.15 Two physical methods for introducing DNA into cells.



Transformation is a mechanism of genetic transfer between bacteria in which the donor DNA exists cell-free in the recipient bacterium's immediate environment. DNA can be naturally released into the environment when cells die and subsequently lyse. Experimentally, DNA containing genes of interest, usually within a plasmid, can be introduced into the environment in order to transform bacterial cells. The ability of a recipient bacterium to take up free DNA and become transformed is known as **competence**. Some strains of bacteria are naturally competent. In others, competence is a brief physiological state during the exponential growth phase; in these bacteria, Ca^{2+} ions enhance the level of competence.

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TRANSFORMATION

- the most common hosts for simple cloning experiment: strains of *E. coli*
- treatment with solutions containing Ca^{2+} ions (sometimes Rb^{+} and Mn^{2+})
  - susceptible to take up exogenous DNA: competent cells

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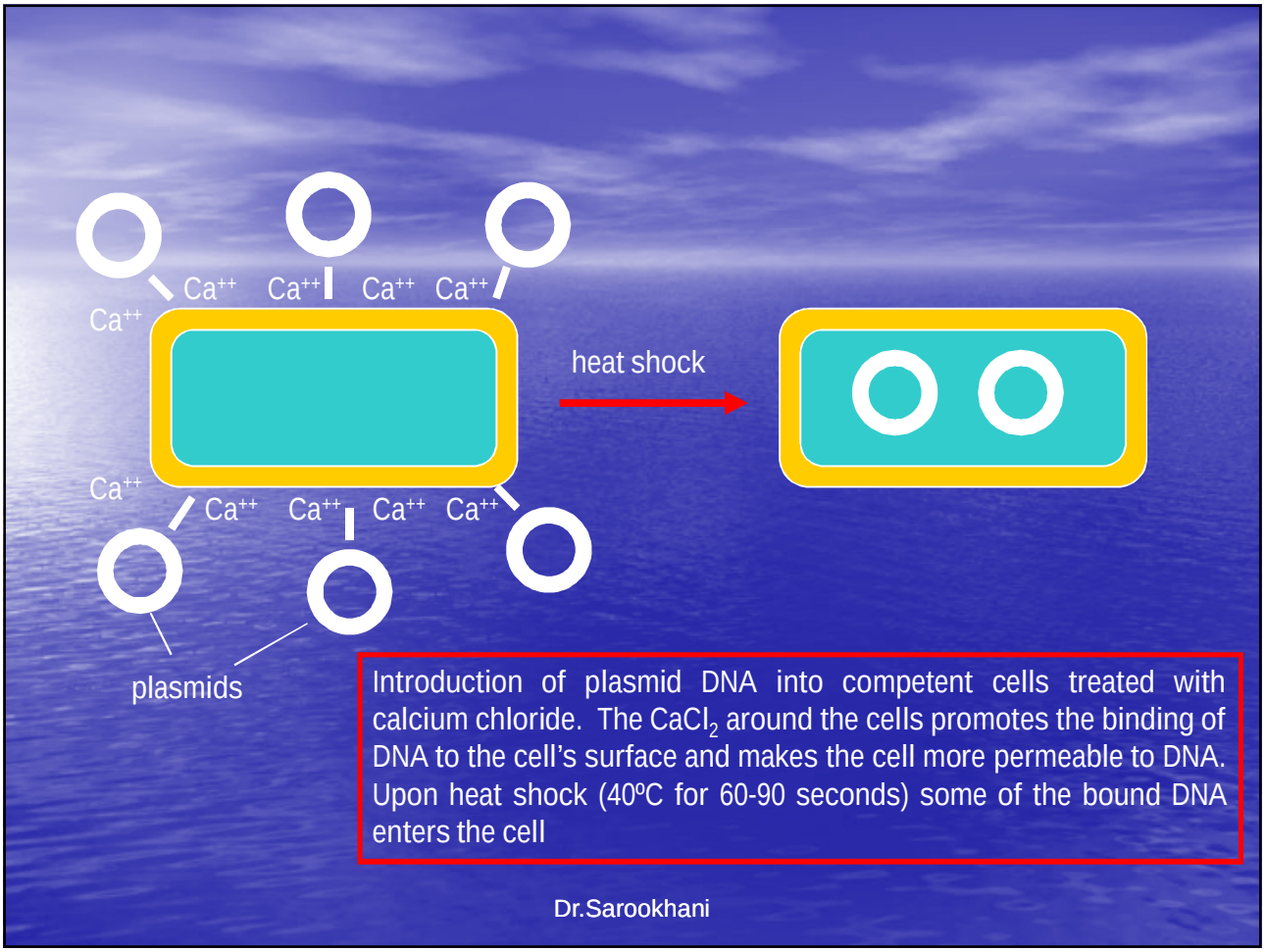
Ca++
Ca++
Ca++
Ca++
Ca++
Ca++
Ca++
Ca++
Ca++
Ca++
heat shock
plasmids
Introduction of plasmid DNA into competent cells treated with calcium chloride. The $CaCl_2$ around the cells promotes the binding of DNA to the cell's surface and makes the cell more permeable to DNA. Upon heat shock (40ºC for 60-90 seconds) some of the bound DNA enters the cell
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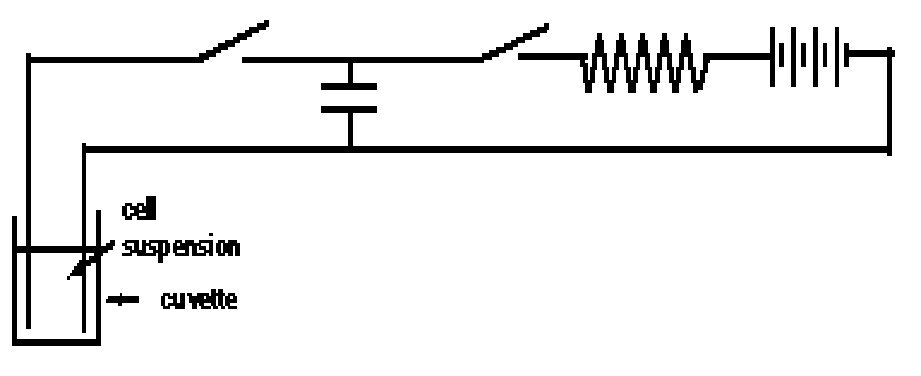
ELECTEROPORATION



•the diagram shows an electrical circuit diagram for a simple electroporation device.
•a cell suspension, such as of plant protoplasts or bacteria, is placed in the cuvette.
•the capacitor is charged by closing the right-hand switch. When the capacitor has been charged, the direct current pulse is discharged in the cuvette suspension by closing the left-hand switch.
•the DC pulse is thought both to disrupt temporarily the membrane and to electrophorese DNA into cells.

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TRANSFECTION

- equivalent to transformation

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SELECTION OF TRANSFORMANTS/MUTANTS

- sensitivity and resistance to chemicals
- requirement for certain compounds for growth (nutrient requirements)
- ability to use (breakdown) compounds
- plaque formation/type
- specific (by nucleic acid hybridization ((southern blotting)) or antibodies (western blotting)))

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INSERTIONAL INACTIVATION

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غير فعال كردن ژن مقاومت به تتراسيكلين پلاسميد pBR322

- جايگاه آنزيم BamHI روي سكانس ژن مقاومت به تتراسيكلين پلاسميد pBR322 قرار دارد چنانچه DNA خارجي در جايگاه اين آنزيم كلون شود و پلاسميد نو تركيب در باكتري ترانسفرم گردد ، باكتري نسبت به تتراسيكلين حساس ميشود. جايگاه آنزيم Pst I روي سكانس ژن مقاومت به آمپی سيلين قرار دارد.

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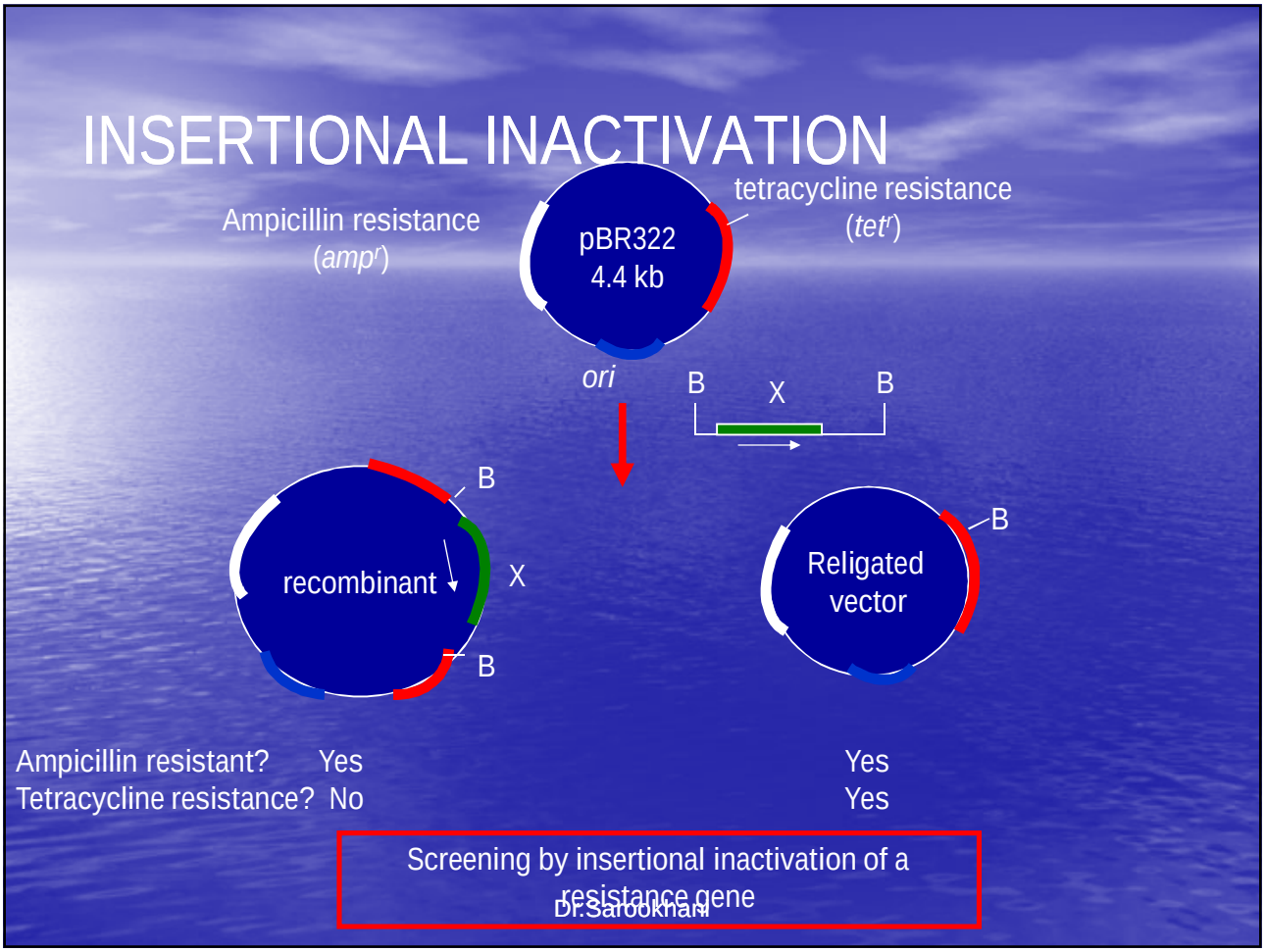
INSERTIONAL INACTIVATION
Ampicillin resistance
(ampr)
pBR322
4.4 kb
tetracycline resistance
(tetr)
ori
B
X
B
recombinant
B
X
B
Religated
vector
B
Ampicillin resistant? Yes
Tetracycline resistance? No
Yes
Yes
Screening by insertional inactivation of a resistance gene
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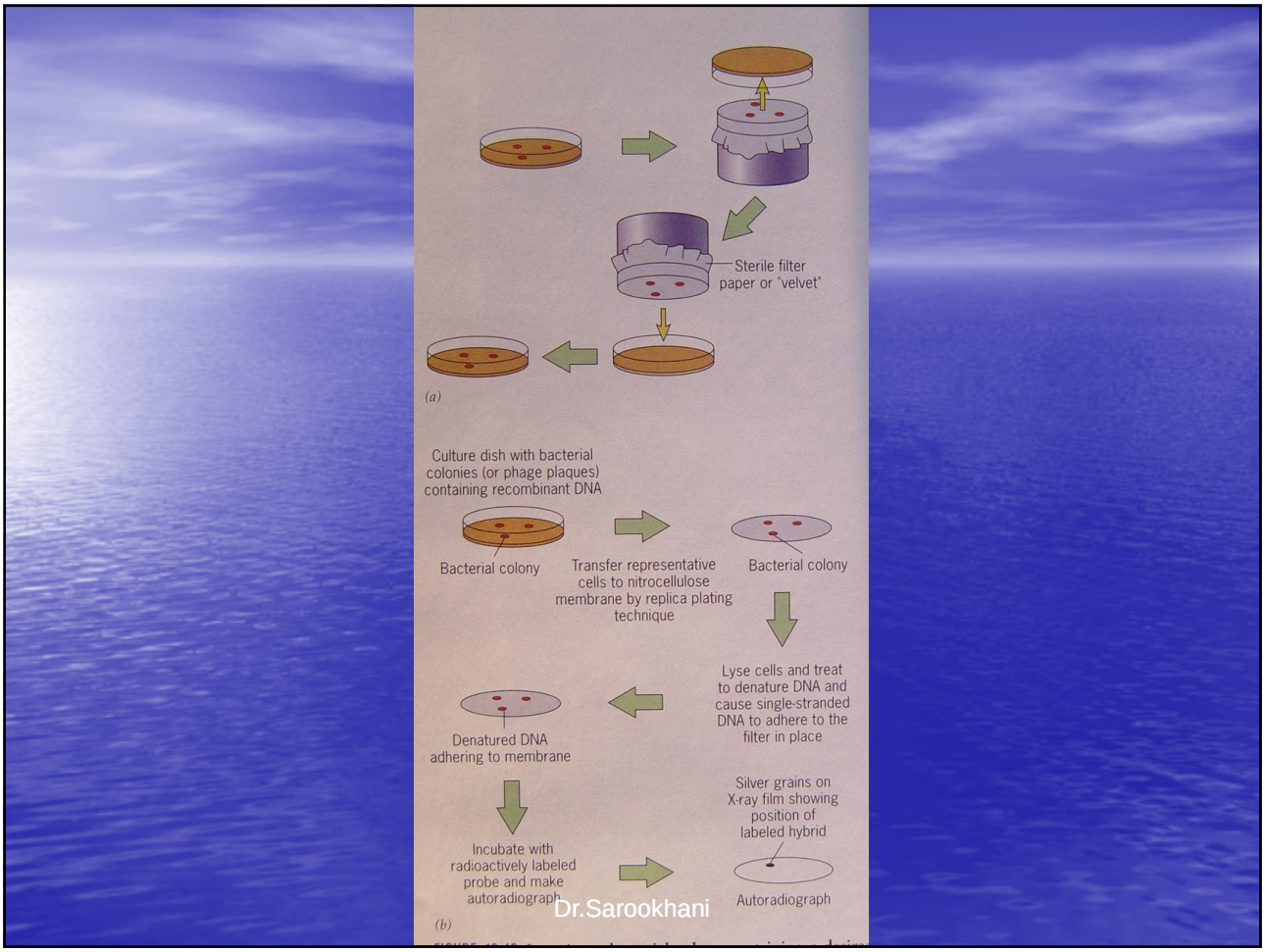
Sterile filter paper or "velvet"
(a)
Culture dish with bacterial colonies (or phage plaques) containing recombinant DNA
Bacterial colony
Transfer representative cells to nitrocellulose membrane by replica plating technique
Bacterial colony
Lyse cells and treat to denature DNA and cause single-stranded DNA to adhere to the filter in place
Denatured DNA adhering to membrane
Incubate with radioactively labeled probe and make autoradiograph
Silver grains on X-ray film showing position of labeled hybrid
Autoradiograph
(b)
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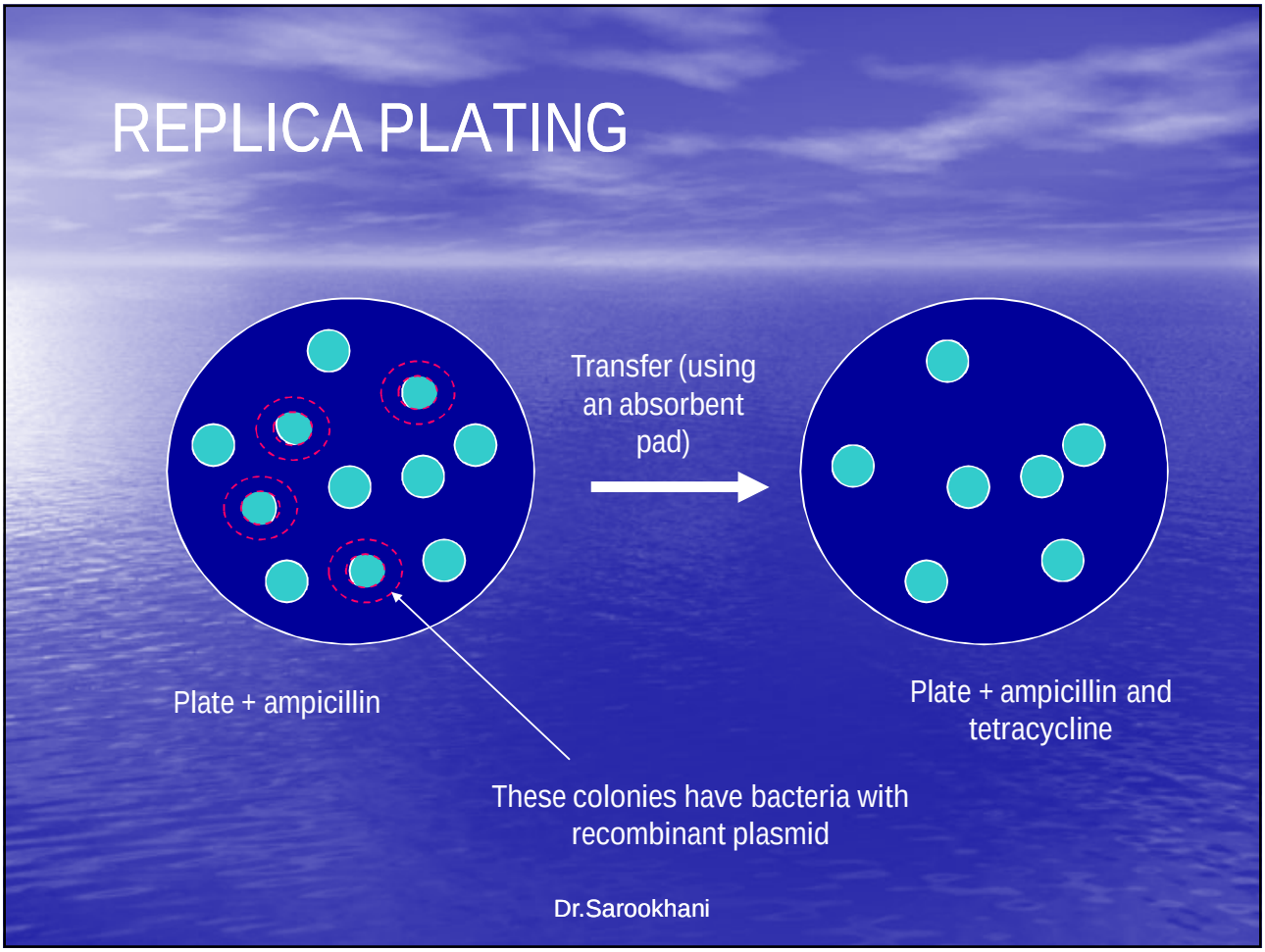
REPLICA PLATING
Transfer (using an absorbent pad)
Plate + ampicillin
Plate + ampicillin and tetracycline
These colonies have bacteria with recombinant plasmid
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SELECTION OF TRANSFORMANTS IN BACTERIOPHAGE

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PLAQUE FORMATION/TYPE

- bacterial lysis and phage release occurs: a clear area is formed (clear plaque)
- lysogeny: turbid plaques

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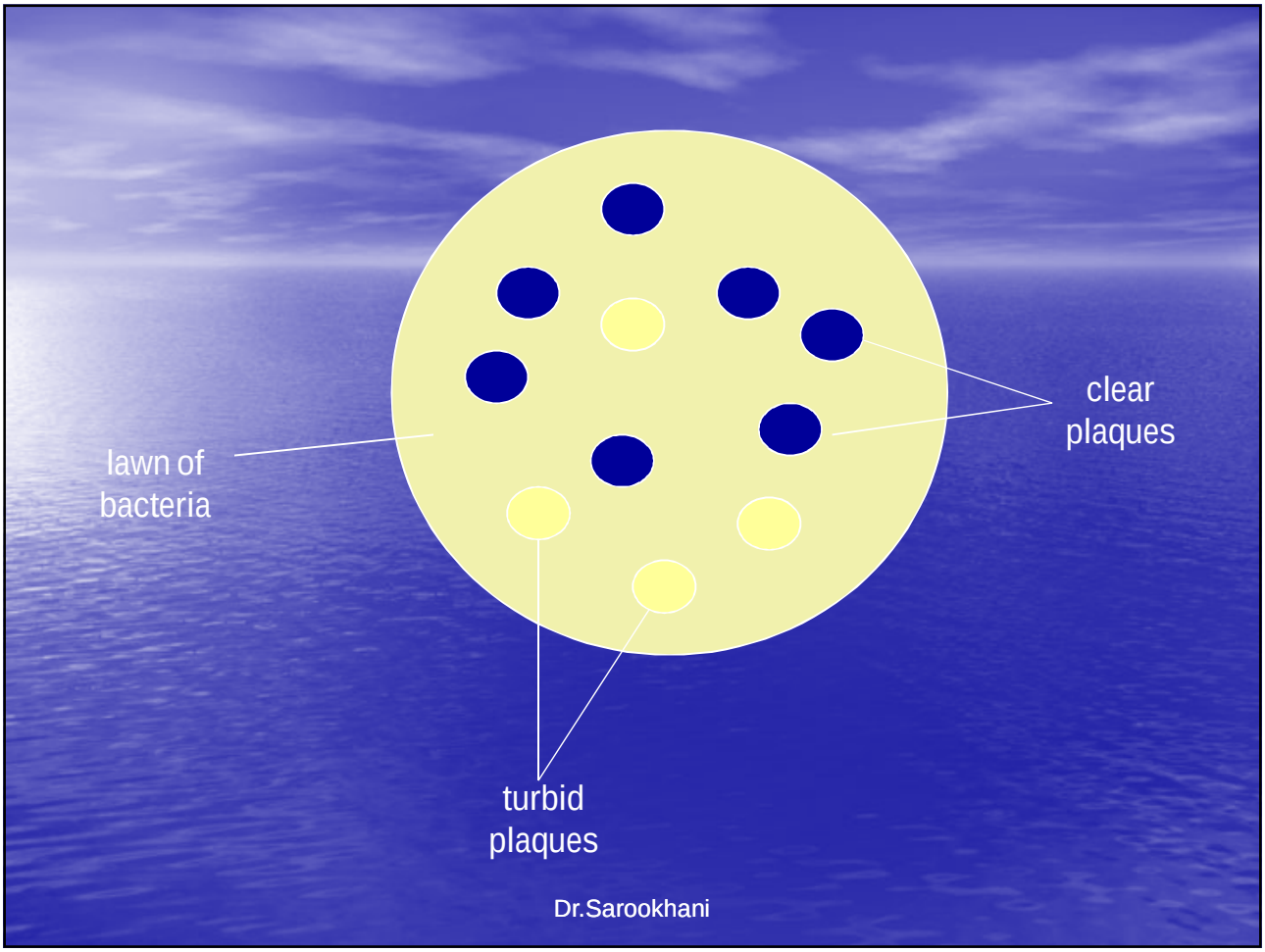
lawn of
bacteria
clear
plaques
turbid
plaques
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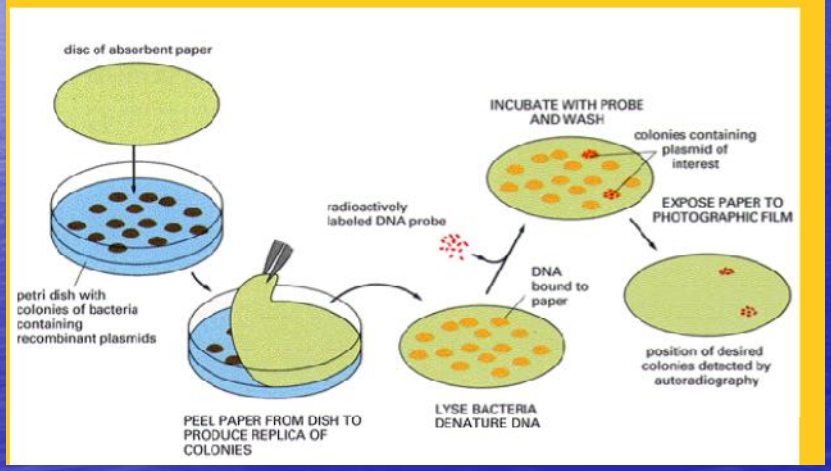
3. Detecting the DNA sequence of a cloned gene with a probe (DNA hybridization)



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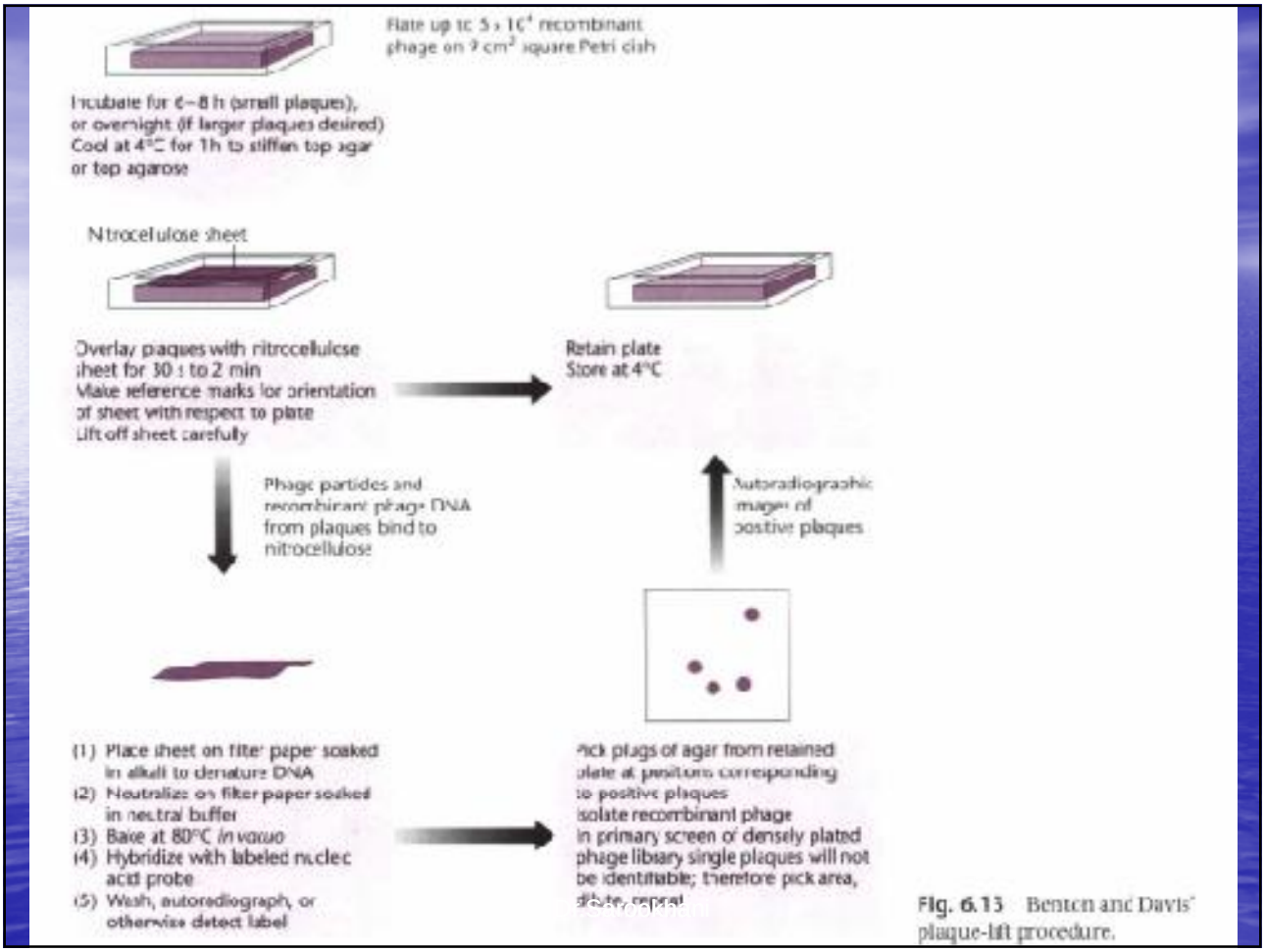
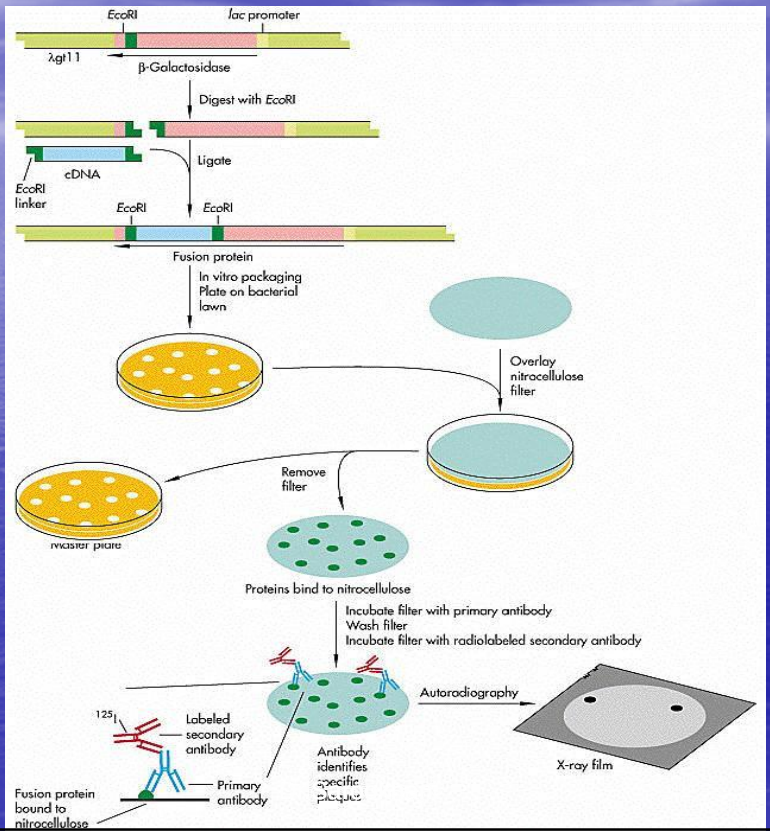


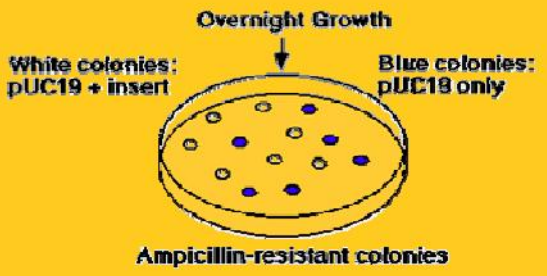
Fig. 6.13 Benton and Davis' plaque-lift procedure.

Western blotting



Screening can involve:

1. Phenotypic screening- the protein encoded by the gene changes the colour of the colony
2. Using antibodies that recognize the protein produced by a particular gene



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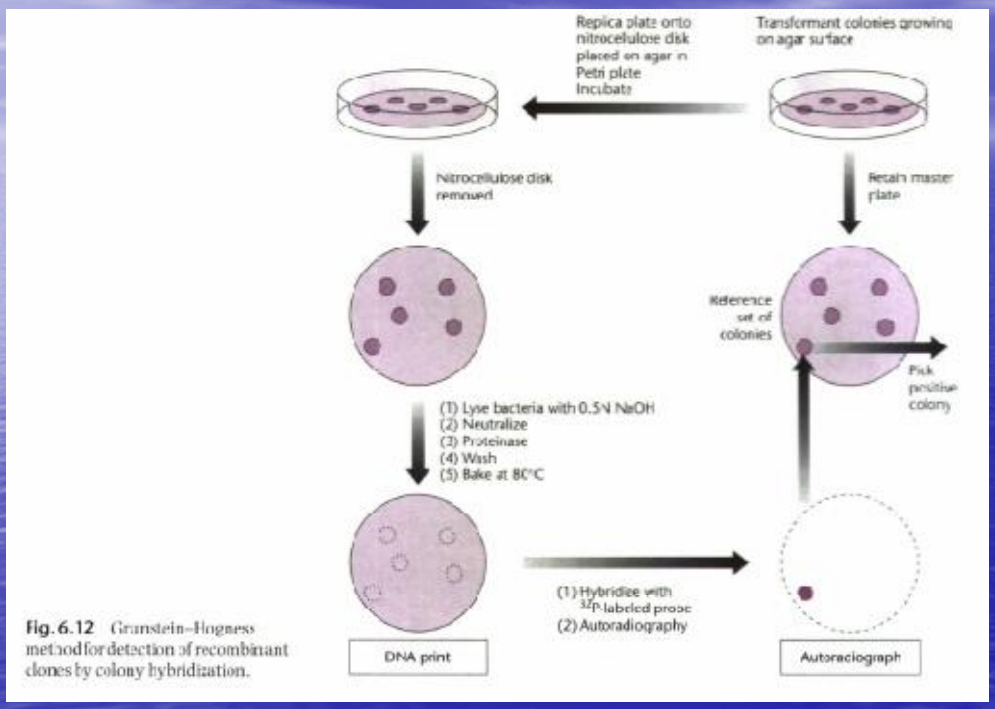

Fig. 6.12 Grunstein–Hogness method for detection of recombinant clones by colony hybridization.

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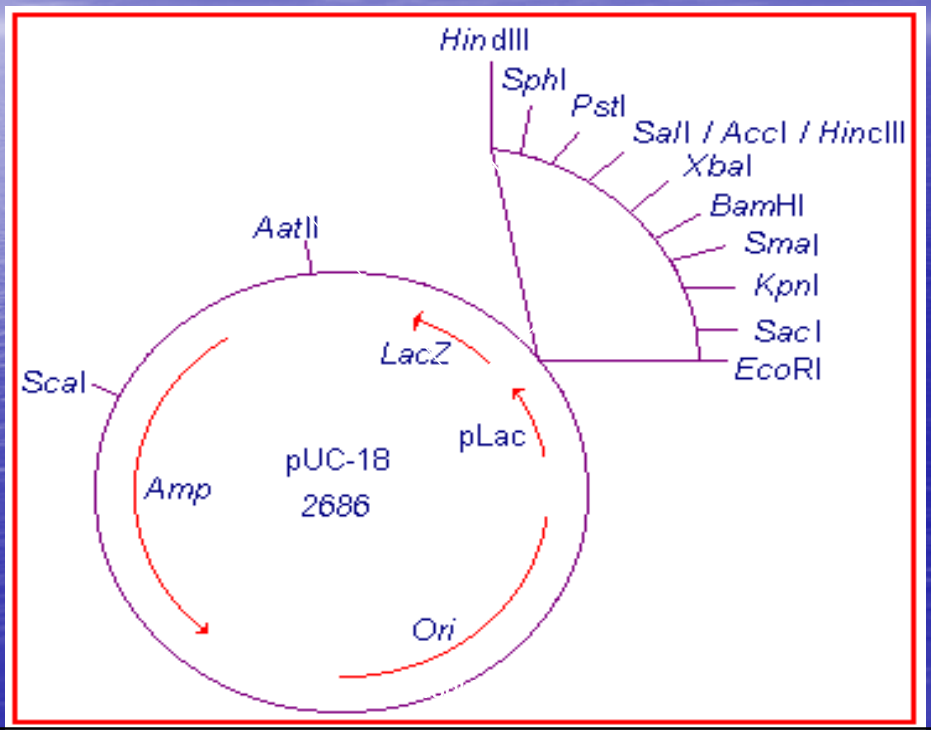
INSERTIONAL INACTIVATION OF LAC Z GENE
(in Lac_. Neg Bacteria)



سيستم Lac selection (Lac Operon)

- سلولي كه حاوي وكتور كلونينگ با يك ژن LacZ` بكر (دست نخورده) است ميتواند بتا گالاكتوزيداز را سنتز كند . اين آنزيم همراه با IPTG (Isopropyl-thiogalactoside)(كه يك القا كننده است) X-gal (برموكلرواينداوليل-گالاكتوپيرانوزيد) را به تركيب آبي رنگ برومو كلرو ايندوليل تبديل ميكند . اين سلولها كلني آبي رنگ (فقط حاوي پلاسميد هستند) يا پلاك آبي رنگ (حاوي فاژ) توليد ميكنند. سلولهاي نوتركيبي كه ژن LacZ` آنها توسط Inserted DNA تخريب شده است نميتوانند بتا گالاكتو زيداز را سنتز كنند و كلني هاي سفيد يا پلاك هاي شفاف توليد ميكنند

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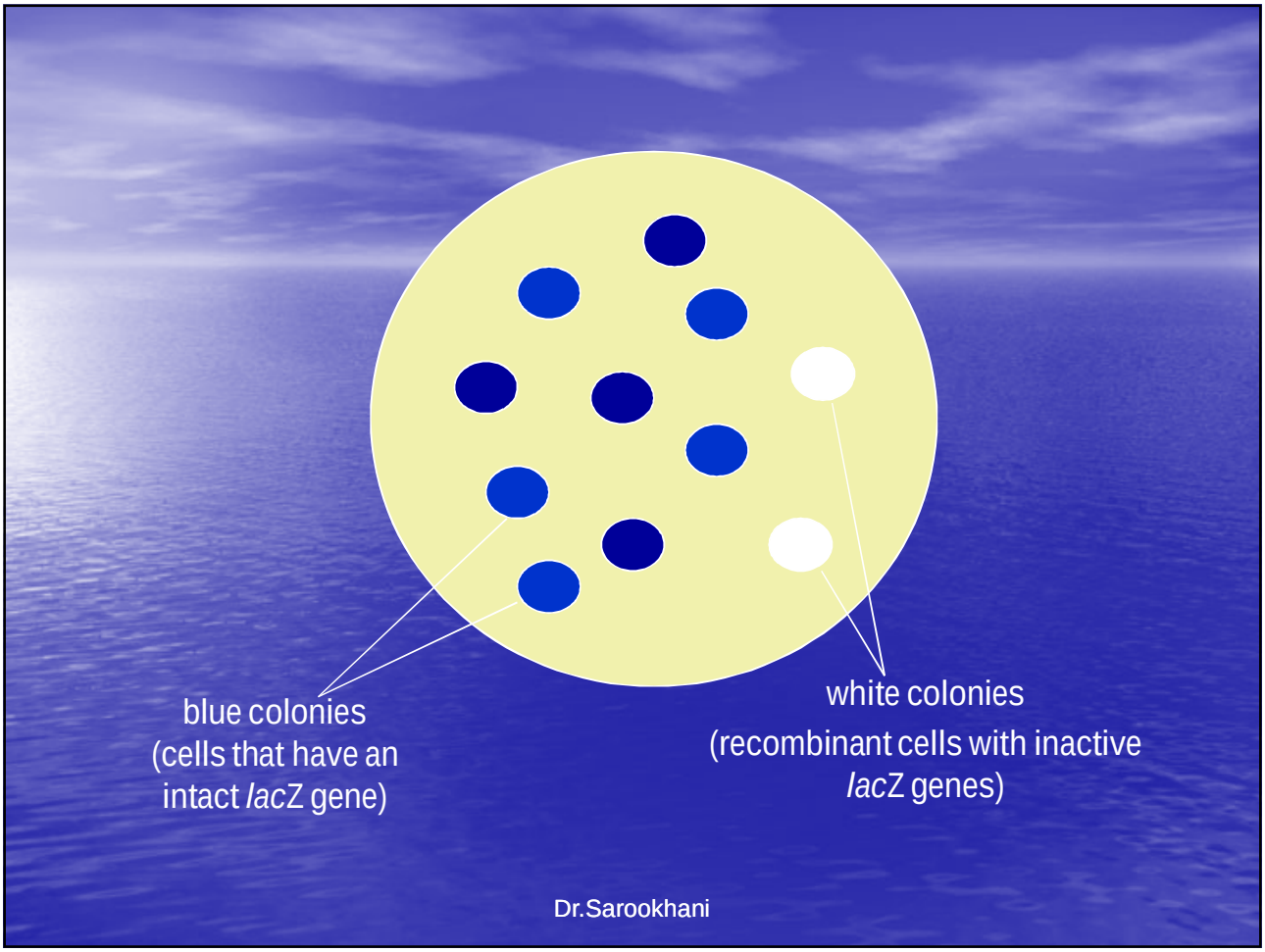
blue colonies
(cells that have an
intact lacZ gene)
white colonies
(recombinant cells with inactive
lacZ genes)
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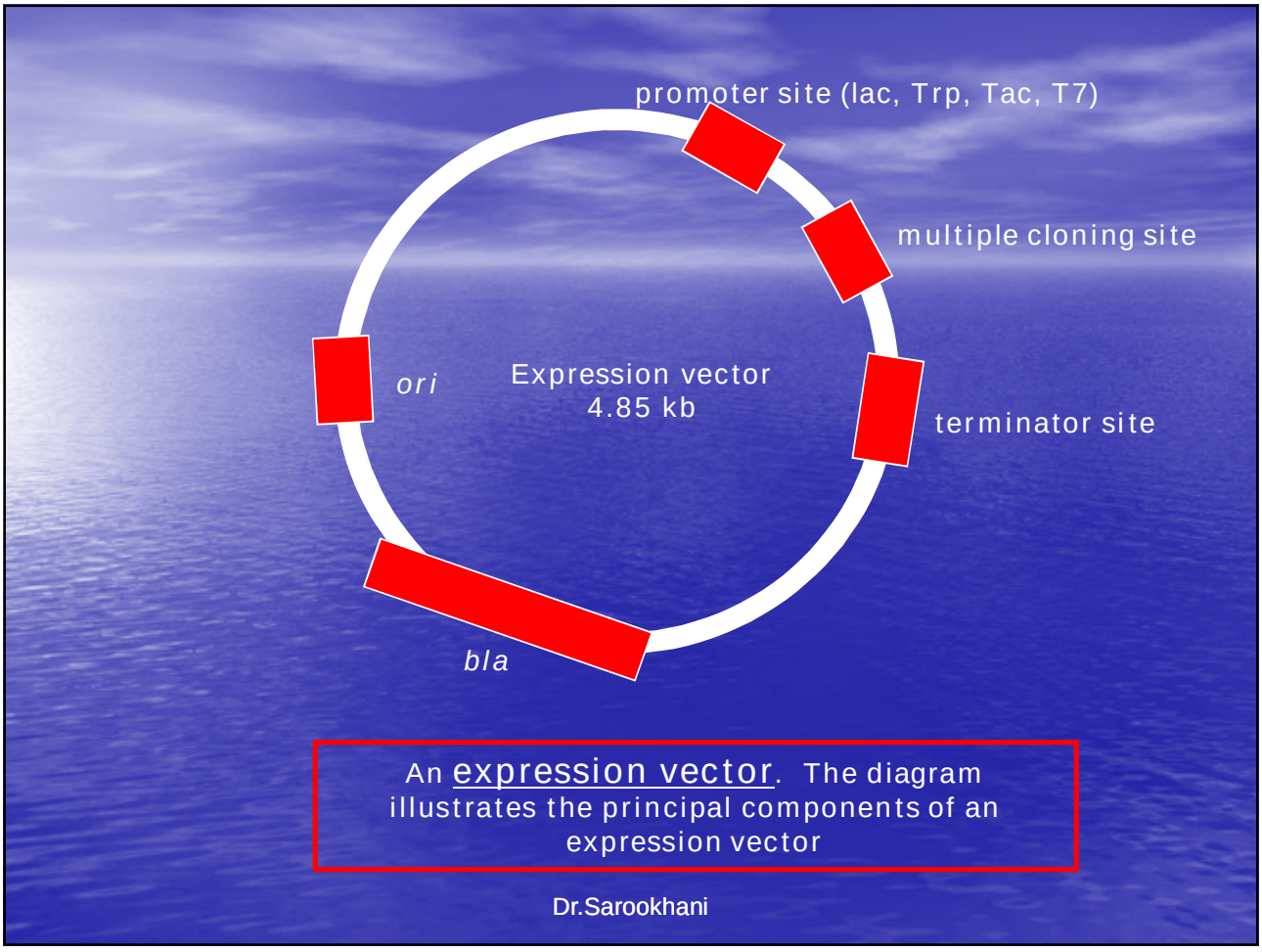

An expression vector. The diagram illustrates the principal components of an expression vector

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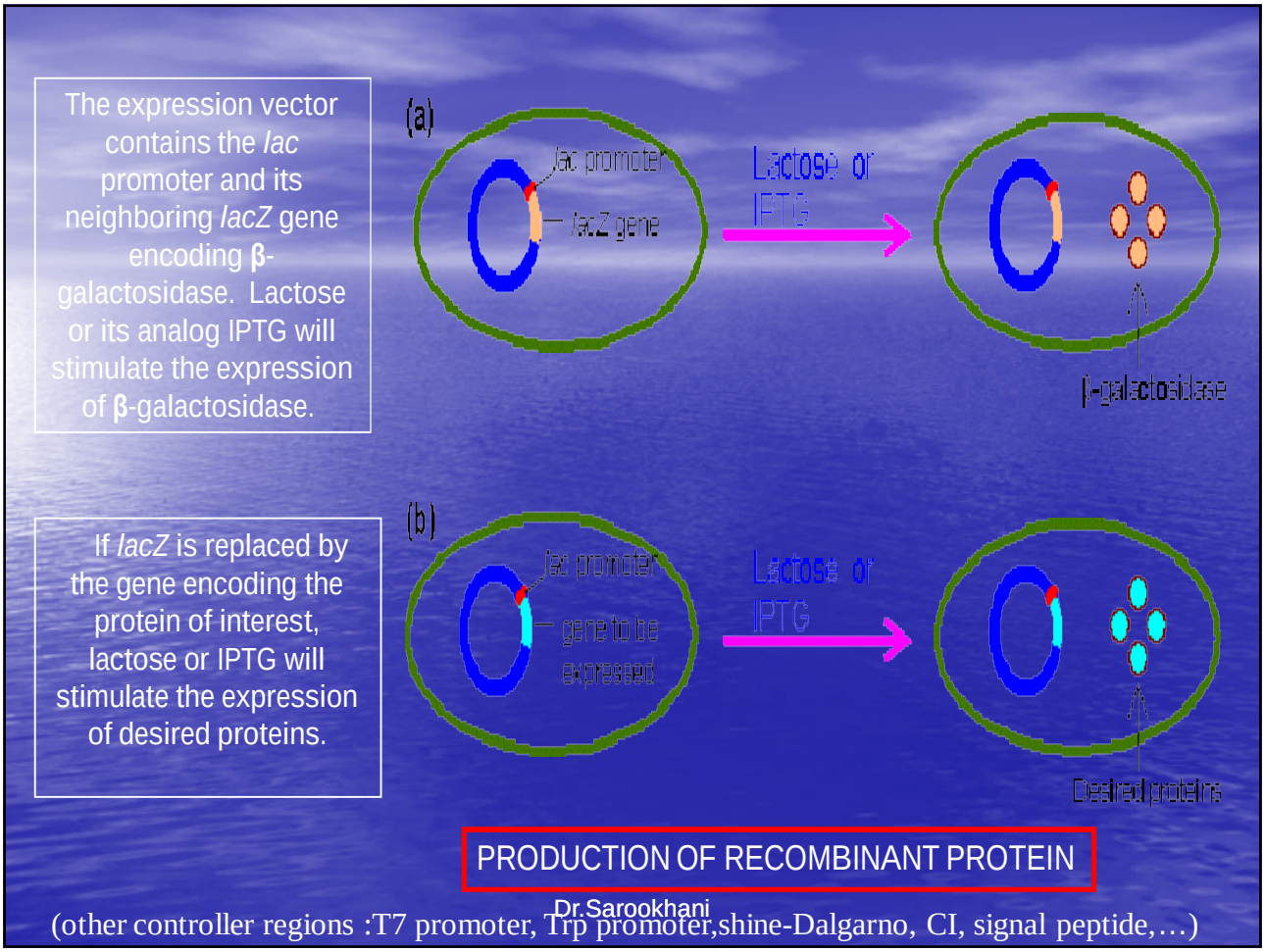
The expression vector contains the *lac* promoter and its neighboring *lacZ* gene encoding β-galactosidase. Lactose or its analog IPTG will stimulate the expression of β-galactosidase.
(a)
lac promoter
lacZ gene
Lactose or IPTG
β-galactosidase
If *lacZ* is replaced by the gene encoding the protein of interest, lactose or IPTG will stimulate the expression of desired proteins.
(b)
lac promoter
gene to be expressed
Lactose or IPTG
Desired proteins
PRODUCTION OF RECOMBINANT PROTEIN
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(other controller regions :T7 promoter, Trp promoter,shine-Dalgarno, CI, signal peptide,…)

1-prepro insulin method
2- separate A & B fragment production

Recombinant INSULIN production